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(54) Title: IMMUNOCAPTURE ASSAY FOR DIRECT QUANTITATION OF SPECIFIC LIPOPROTEIN CHOLESTEROL LEVELS

## (57) Abstract

The present invention relates to a method for directly measuring concentrations of cholesterol associated with specific lipoproteins in a plasma sample. The method involves the specific capture of intact lipoprotein particles containing cholesterol from a plasma sample with a specific lipoprotein binding agent. The quantity of the specific lipoprotein cholesterol present in the sample is then measured by detecting the amount of binding-agent-lipoprotein complexes that have formed in the reaction. The cholesterol contained in the binding-agent-lipoprotein complexes can be detected by reacting the complexes with labeled cholesterol specific binding agents and measuring the amount of label bound thereto, or by releasing the cholesterol in the complexes and measuring the amount of cholesterol released. The specific lipoprotein binding agent can be bound to a solid support. The assay method may also incorporate a further step of separating the solid support from the sample before detecting the presence of cholesterol bound to the solid support.

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concentration by dividing the triglyceride concentration by 5 Although these assumptions may not be strictly true, the equation generally provides LDL-cholesterol values within a few mg/dL of those measured by the ultracentrifugation method. However, large errors in the calculated LDL-cholesterol concentration occur in the samples with triglycerides exceeding 400 mg/dL. In such cases, the Friedewald calculation method is unacceptable. Several studies have been conducted for the purpose of modifying Friedewald's equation so that the calculated LDL-cholesterol would better correlate with the ultracentrifugation method (McNamara et al. (1990) *Clin. Chem.* 36:36-42; Warnick et al. (1990) *Clin. Chem.* 36:15-19; Delong et al. (1986) *JAMA* 256:2372-2377), but no significant improvement has been made. Like the ultracentrifugation method, the LDL-cholesterol estimated by Friedewald's equation also contains contributions from IDL cholesterol and Lp(a)-cholesterol. Despite these limitations, both of the above methods are commonly used. For example, in most of the epidemiological studies wherein the relationship between LDL-cholesterol and CHD was established (Bachorik (1989) *Clin. Lab. Med.* 9:61-72; Castelli et al. (1986) *JAMA* 256:2823-2828) as well as in studies of LDL-cholesterol lowering drugs (Stamler et al. (1986) *JAMA* 256:2823-2828; Frick et al. (1987) *N. Engl. J. Med.* 317:1237-1245; Blankenhorn et al. (1987) *JAMA* 257:3233-40; Lipid Research Clinics Program (1984) *JAMA* 251:351-374), one or both of these methods were used. Thus, what is generally called "LDL-cholesterol" is actually a measure of LDL-cholesterol, IDL cholesterol and Lp(a)-cholesterol, each of which are thought to be atherogenic markers. Moreover, Lp(a)-cholesterol concentration in plasma is independent of total cholesterol, HDL-cholesterol or triglycerides. Thus, the measurement of Lp(a) should be done independently (Kurchinski et al. (1989) *Clin. Chem.* 35:2156-2157). A method for estimating Lp(a)-cholesterol concentration involves measuring the total Lp(a) mass in plasma ([Lp(a)]) and calculating the Lp(a)-cholesterol

Because the unfractionated plasma layer ([d >1.006 g/mL chol]) also contains IDL and Lp(a) in addition to LDL and HDL, the LDL-cholesterol content of this fraction ([LDL chol]) represents contributions from LDL, IDL, and Lp(a). In most cases, the contributions of remnants, IDL- and Lp(a)-cholesterol are only a few mg/dL and in all past epidemiological studies, their contributions were ignored. However, recent studies have indicated that IDL and Lp(a) are atherogenic markers. Elevated Lp(a) and IDL levels have been correlated with CHD and the progression of atherosclerotic lesions (Rhoads et al. (1986) *JAMA* 256:2540-2544; Guyton et al. (1985) *Arteriosclerosis* 5:265-272; Schriewer et al. (1984) *J. Clin. Chem. Clin. Biochem.* 22:391-396; Albers et al. (1974) *Lipids* 9:15-26; Albers et al. et al.(1974) *Biochem. Genetics* 11:475-486; Tatami et al. (1981) *Circulation* 64:1174-1784; Krauss et al. (1987) *Lancet* 2:62-66).

The ultracentrifugation method of LDL-cholesterol quantitation is time consuming, expensive, and requires specialized equipment, facilities and laboratory skills. A second method is more commonly used which simplifies this process. In this method the plasma LDL-cholesterol concentration is estimated by measuring three separate cholesterol concentrations: total-cholesterol ([Total-chol]), HDL-cholesterol ([HDL-chol]), and triglycerides concentration in the plasma. The LDL-cholesterol ([LDL-chol]) plasma concentration is then calculated using Friedewald's equation (Friedewald et al. (1972) Clin. Chem. 18:499-502) as follows:

$$[\text{LDL-chol}] = [\text{Total-chol}] - [\text{HDL-chol}] - [\text{Triglycerides}/5].$$

The factor [Triglyceride/5] relates to the plasma VLDL-cholesterol concentration. It is assumed that all plasma triglycerides are associated with VLDL and that the ratio of triglyceride concentration to cholesterol concentration associated with VLDL is about 5. Thus, the VLDL-cholesterol concentration can be calculated from the triglyceride

very low density lipoprotein (VLDL, d<1.006 g/mL), low density lipoprotein (LDL, d 1.019-1.063 g/mL) and high density lipoprotein (HDL, d 1.063-1.21 g/mL). Lesser amounts of cholesterol are also carried out in two minor lipoprotein classes, intermediate density lipoprotein (IDL, d 1.006-1.019 g/mL) and lipoprotein (a) (Lp(a), d 1.045-1.080 g/mL). LDL is the major contributor to the plasma total cholesterol concentration in man, accounting for the one-half to two-thirds of the plasma cholesterol.

Two methods are presently available to determine the quantitative association between LDL-cholesterol and CHD. A first method, generally referred to as ß-quantitation, is based on the use of combined ultracentrifugation and polyanion precipitation procedures (Havel et al. (1955) *J. Clin. Invest.* 34:1345-1353; McNamara et al. (1990) *Clin. Chem.* 36:36-42; Warnick et al. (1982) *Clin. Chem.* 28:1379-1388). In this method, an aliquot of plasma is used to measure the total cholesterol concentration in the sample. A second aliquot of plasma is centrifuged (105,000 x g) at a plasma density of 1.006 g/mL for 18 hours at 4°C. After centrifugation, the upper layer containing VLDL and chylomicrons is removed. Chylomicrons (d<1.006 g mL) are microscopic lipid particles that appear in the blood transiently after a fat-containing meal and are rich in triglycerides and usually have no significant effect on the total- and LDL-cholesterol concentration. An aliquot of the remaining bottom layer, which contains LDL, IDL, Lp(a) and HDL, is then used to measure the cholesterol concentration ([d >1.006 g/mL chol]) in this unfractionated plasma layer. LDL, VLDL, IDL and Lp(a) are precipitated from a second aliquot of the unfractionated plasma layer (Bachorik et al. (1986) Methods in Enzymol. 129 (B) 78-100) and the cholesterol concentration of HDL ([HDL-chol]) in the supernatant is measured. The cholesterol concentration of LDL ([LDL chol]) is then calculated using the following equation:

$$[\text{LDL chol}] = [\text{d} > 1.006\ \text{g/mL chol}] - [\text{HDL-chol}].$$

# IMMUNOCAPTURE ASSAY FOR DIRECT QUANTITATION OF SPECIFIC LIPOPROTEIN CHOLESTEROL LEVELS

This application is a Continuation-In-Part of Application Serial No. 07/847,502, filed March 6, 1992.

## BACKGROUND OF THE INVENTION

Total plasma cholesterol is known to be an unreliable marker for prediction of coronary heart disease (CHD) in many patients. Epidemiological studies have established several lipoprotein-related risk factors for coronary heart disease. Elevated plasma levels of cholesterol associated with low density lipoprotein (LDL) markedly increase the risk of CHD (Castelli et al. (1986) JAMA 256:2835-2838). Lowering plasma LDL-cholesterol concentrations reduces the risk of CHD, myocardial infarction (MI) and CHD-related death (Lipid Res. Clinics Program (1984) JAMA 251:351-374; Frick et al. (1987) N. Engl. J. Med. 317:1237-1245; Blankenhorn et al. (1987) JAMA 257:3233-3240; Stamler et al. (1986) JAMA 256:2823-2828). Moreover, reducing the plasma LDL-cholesterol concentration in patients also reduces the incidence of second heart attacks in MI survivors, slows down the progression of CHD and may also lead to regression of coronary atherosclerosis (Brenski et al. (1984) 69:313-324). The National Cholesterol Education Program (NCEP) published recommendations for the detection and treatment of high blood cholesterol in adults for total- and LDL-cholesterol (Recommendations for improving cholesterol measurement (1990) Bethesda:NIH Publication No. 90-2964). The recommended ranges for LDL-cholesterol are: <130 mg/dL (desirable); 130-159 mg/dL (borderline high); ≥160 mg/dL (high). No reliable method for the direct quantitation of LDL is yet available (Friedewald et al (1972) Clin. Chem. 18:499-502; Warnick et al (1982) Clin. Chem. 28:1379-1388.

In the fasting state, plasma cholesterol is normally transported primarily in three major lipoprotein fractions,

concentration from the total Lp(a) mass concentration. The Lp(a)-cholesterol concentration is assumed to be about 30% of the total Lp(a) mass concentration (Kostner et al. (1981) *Atherosclerosis* 38:51-61). The calculated LDL-cholesterol concentration then can be corrected for Lp(a)-cholesterol using one of the following equations:

[LDL-chol] =
[Total-chol] - [HDL-chol] - [Triglyceride/5] - 0.3 [Lp(a)]
or
[LDL-chol] =
[d>1.006 g/ml chol] - [HDL-chol] - 0.3 [Lp(a)]

(Jurgens et al. (1987) *Neurology* 37:513-515; Sandkamp et al. (1990) *Clin. Chem.* 36:20-23). Alternatively, the Lp(a) concentration can be calculated from the plasma Lp(a)-protein concentration. The Lp(a)-protein concentration was measured by an ELISA test and the Lp(a) concentration was calculated by multiplying the Lp(a)-protein concentration by 4.21 (Fless et al. (1989) J. Lipid Res. 30:651-662).

It is important that the Lp(a)-cholesterol correction be made in the LDL-cholesterol concentration because studies have shown that diet and drug treatment will reduce LDL-cholesterol levels but not Lp(a)-cholesterol levels and proper patient monitoring requires an accurate measurement of LDL-cholesterol levels (Albers et al. (1975) *Metabolism* 24:1047-1054; Vessby et al. (1982) *Atherosclerosis* 44:61-71). This is particularly true for patients with elevated levels of Lp(a). For example, the Lp(a) concentration in some patients have been found to be as high as 100 mg/dL (i.e., 30 mg/dL cholesterol) (Fless et al. U.S. Patent (1990) 4,945,040). In such patients, the LDL-cholesterol values will be erroneous if no correction is made for Lp(a)-cholesterol.

Direct methods for LDL-cholesterol measurement have been reported in the literature, but they involve selective precipitation of LDL from plasma. Three commercial kits (Boehringer Kit, Merck Kit, and Bio Merieux Kit) are available

and have been evaluated by Mulder. et al. ((1984) *Clin. Chim. Acta.* 143:29-35). The Boeringer Kit (Boeringer Mannheim, Federal Republic of Germany) uses selective precipitation of LDL with polyvinyl sulfate. The cholesterol content in the supernatant is assayed and subtracted from the total serum cholesterol to obtain the LDL-cholesterol concentration. The Merck Kit (Merck, Darmstadt, Federal Republic of Germany) uses selective precipitation of LDL by heparin at pH 5.12. As in the Boeringer Kit, the LDL-cholesterol concentration is calculated by subtraction of supernatant cholesterol from total cholesterol. The Bio Merieux Kit (Bio Merieux, Marcy-l'Etoile, France) uses selective precipitation of LDL with polycyclic surface active anions. After centrifugation and removal of the supernatant, the cholesterol content of the precipitate is measured (Eur. Patent (1982) 0076211A2).

All three commercial methods agree well with isolated LDL fractions (d 1.019-1.063 g/ml) for samples with triglyceride concentrations below about 180 mg/dL but significantly overestimate the LDL-cholesterol concentration for samples with higher triglyceride concentrations. However, the Mulder, et al. study cannot be compared directly with other studies because the isolated LDL fractions (d 1.019-1.063 g/mL) do not contain IDL (d 1.006-1.019 g/mL) and only contain a portion of Lp(a) that may have been present (Lp(a) ≈ d 1.045-1.080 g/mL). No direct comparison of these kits was made with the ultracentrifugation-polyanion precipitation method. Also, the presence of lipoproteins other than LDL in the precipitate was not investigated.

Another method for direct LDL-cholesterol measurement (Kerscher et al. (1988) U.S. Patent 4,746,605) involves the immunoprecipitation of HDL with HDL-specific antibodies and precipitation of VLDL with a mixture of polyanion and divalent cation. The location and effect of Lp(a) and IDL in the assay was not determined. Other precipitation or agglutination methods useful in direct LDL measurement have been reported, such as cross-linked sulfated polyvinyl alcohol (Maaskant et al. (1986) U.S. Patent 4,623,628) as a binder for LDL, a water-

insoluble anion-exchanger which binds to VLDL and HDL (Maier et al. (1986) U.S. Patent 4,569,917), Ricinus Communis lectin as a agglutinating agent for LDL (Sears (1980) U.S. Patent 4,190,628), surfaces coated with anionic groups which have affinity for LDL (Knox et al. (1988) Eur. Patent 0319250A1), but none of these methods or reagents were evaluated with clinical samples.

Methods for the direct measurement of apolipoprotein B (apo B) utilizing apo B-specific antibody have been reported. Such methods include competitive fluid phase and solid phase radio-immunoassays (RIA), an enzyme-linked immunosorbant assay (ELISA), a radial immunodiffusion assay (RIDA), an electroimmunoassay (EA), and an immunoprecipitation assay (IPA). However, the antisera used in these assays lacked sufficient specificity to make the assays useful or reproducible. The methodological problems of each of these assays have been reviewed (Labeur et al. (1990) *Clin. Chem.* 36:591-597; Curry et al. (1978) *Clin. Chem.* 24:280-286).

Other methods that have been used for directly measuring LDL-cholesterol include separation and quantitation by high performance liquid chromatography (HPLC), gel filtration chromatography, analytical ultracentrifugation, sequential-density-gradient or zonal ultracentrifugation and electrophoresis. ("Methods in Enzymol." (1986) Vol. 128, Part A; Vol. 129, part B). These methods generally require specialized skills and expensive equipment and are not readily adapted for use in routine analysis of patient samples.

An object of this invention is to develop a method of measuring LDL-cholesterol directly. Another object of this invention is to eliminate the presence of IDL- and Lp(a)-cholesterol in the LDL-cholesterol measurement. Another object of this invention is to directly measure LDL-cholesterol easily, cheaply, quickly, and accurately without the need of highly trained technicians or expensive equipment such as an ultracentrifuge. Still another object of this invention is to reduce the number of analytes and steps that are presently necessary to measure the LDL-cholesterol concentration. Yet

another object of this invention is to directly measure LDL-cholesterol without the analytical variability generally associated with LDL-cholesterol measurement.

While the role of Lp(a) in CHD has not been fully investigated, a significant correlation between elevated Lp(a) serum or plasma levels in humans, coronary artery disease and the progression of atherosclerotic lesions has been established (Seed et al. (1990) New England J. Med. 332:1494-1499). Lp(a) and LDL have common structural features. Both have a similar lipid composition and an apo B component. Lp(a), however, also contains two molecules of apolipoprotein (a) (apo(a)) covalently linked to the apo B molecule by at least one sulfide bond. Lp(a) concentrations in human plasma range from 1 mg/dL to more than 100 mg/dL. Lp(a) is known to be transmitted genetically by an autosomal dominant trait and 20 to 35% of normal individuals have Lp(a) plasma concentrations greater than 30 mg/dL. Lp(a) levels appear to be insensitive to changes in diet (Albers, et al. (1977) J. Lipid Res. 18:331-338) and treatment with cholestyramine (Vessby, et al. (1982) Atherosclerosis 44:61-71). Neomycin and niacin have been shown to reduce Lp(a) levels by 45% (Gurakar, et al. (1985) Atherosclerosis 57:293-301). However, the effect of diet and lipid-lowering drugs on the Lp(a)-cholesterol levels is not known. Another object of the present invention is to measure the plasma Lp(a)-cholesterol concentration of patients easily and accurately and thus allowing researchers to further investigate the relationship between Lp(a)-cholesterol concentrations and CHD.

A number of assay methods for quantating Lp(a) in plasma are known (Morisett, et al. (1987) "Plasma Lipoproteins", A.M. Gottto, Jr., ed., Elsevier Science B.V., Chapter 5, pp. 129-152; Gaubatz, et al. (1986) "Methods in Enzymol." Vol. 129, pp. 167-187). The assays include radioimmunoassays, enzyme-linked immunosorbent assays, radial immunodiffusion, electroimmunoassays and immunoelectrophoresis. The assays commonly use antibodies directed against the apo(a) components of Lp(a) to measure

total Lp(a) or its protein content (Albers, et al. (1990) Clin. Chem. 36:2019-2026). These methods generally require trained technicians to perform these time-consuming methods and are not readily adaptable to routine analysis of patient samples. Moreover, none of these methods can quantitate the cholesterol content of Lp(a) directly from the patient sample. To quantitate the cholesterol concentration associated with Lp(a) in a patient sample, at a minimum the Lp(a) would have to be physically separated from the sample, such as by chromatographic or ultracentrifugal techniques, prior to measurement of the cholestrol concentration. Thus, another object of the present invention is to directly measure the Lp(a)-cholesterol concentration easily, cheaply, quickly, and accurately without the need of highly trained technicians or expensive equipment such as an ultracentrifuge.

## SUMMARY OF THE INVENTION

The present invention relates to a method for directly measuring concentrations of cholesterol associated with specific lipoproteins in a plasma sample. The method involves the specific capture of intact lipoprotein particles containing cholesterol from a plasma sample with a specific lipoprotein binding agent. The quantity of the specific lipoprotein cholesterol present in the sample is then measured by detecting the amount of binding-agent-lipoprotein complexes that have formed in the reaction. The cholesterol contained in the binding-agent-lipoprotein complexes can be detected by a variety of standard methods known in the art such as, enzyme immunoassays, radioimmunoassays, ELISA, EMIT, and the like, or with labeled cholesterol specific binding agents. The specific lipoprotein binding agent can be bound to a solid support. The assay method may also incorporate a further step of separating the solid support from the sample before detecting the presence of cholesterol bound to the solid support. The present invention is also directed to a method

for selecting anti-LDL specific antibodies that are useful for detecting the amount of LDL-cholesterol in a sample.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Typical antibody titer plots of the monoclonal antibody MB16 obtained by incubating microtiter plates with LDL, VLDL, IDL, HDL and Lp(a) bound to the plates in separate wells and measuring the antibody bound to the lipoproteins by an ELISA assay.

Figure 2: Typical competitive binding curves of the monoclonal antibody MB16 obtained by pre-incubating the antibody with a lipoprotein, adding the mixture to a microtiter plate with LDL bound to the plate reaction wells and measuring the antibody bound to the LDL by an ELISA assay.

Figure 3: Typical binding curves of the HRPO labeled MAB B06 with lipoproteins LDL, VLDL, IDL, HDL and Lp(a) as described in Example 3.

Figure 4: Typical binding curves of MABs 4B5.6, SPL4A5, 8A2.1, and 465C3D1 bound to microtiter plates for LDL-cholesterol using HRPO labeled MAB B06.

Figure 5: Typical binding curves of 125I-LDL to MABs 2D8, 1D1 and MB16 immobilized on Immulon 2 Removawell strips.

Figure 6: Typical competitive displacement curves of 125I-LDL for MAB 4B5.6 with lipoproteins LDL, VLDL, IDL, HDL and Lp(a).

Figure 7: A is a chart summarizing the composition of the two antigenic epitopes of apoB. B is a peptide map of the apo B fragments formed by thrombin.

Figure 8: Typical peptide fragments of the apo B T2 fragment which are useful in generating LDL-specific MABs.

Figure 9: A typical cholesterol standard curve for a specific LDL-cholesterol assay of this invention.

Figure 10: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB SPL4A5-Sepharose and the ultracentrifuge method. B is a

correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB SPL4A5-Sepharose and the Friedewald method.

Figure 11: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 8A2.1-Sepharose and the ultracentrifuge method. B is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 8A2.1-Sepharose and the Friedewald method.

Figure 12: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifuge method. B is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 4B5.6-Sepharose and the Friedewald method.

Figure 13: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifuge method corrected for Lp(a)-cholesterol. B is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB 4B5.6-Sepharose and the Friedewald method corrected for Lp(a)-cholesterol.

Figure 14: A is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifugation method to the triglyceride concentration. B is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifugation method corrected for Lp(a)-cholesterol to the triglyceride concentration.

Figure 15: A is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifugation method to the VLDL-cholesterol concentration. B is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB 4B5.6-Sepharose and the ultracentrifugation method

corrected for Lp(a)-cholesterol to the VLDL-cholesterol concentration.

Figure 16: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifuge method. B is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB MB16-Sepharose and the Friedewald method.

Figure 17: A is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifuge method corrected for Lp(a)-cholesterol. B is a correlation curve for LDL-cholesterol measurements by the immunocapture assay using MAB MB16-Sepharose and the Friedewald method corrected for Lp(a)-cholesterol.

Figure 18: A is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifugation method to the triglyceride concentration. B is a correlation curve for the ratio of LDL-cholesterol concentrations, determined by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifugation method corrected for Lp(a)-cholesterol to the triglyceride concentration.

Figure 19: A is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifugation method to the VLDL-cholesterol concentration. B is a correlation curve for the ratio of LDL-cholesterol concentrations determined by the immunocapture assay using MAB MB16-Sepharose and the ultracentrifugation method corrected for Lp(a)-cholesterol to the VLDL-cholesterol concentration.

Figure 20: A correlation curve for LDL-cholesterol measurements by the dry immunocapture assay using MAB 4B5.6 as described in Example 12 and the Friedewald method.

Figure 21: A correlation curve for LDL-cholesterol measurements by the indirect immunocapture assay using MAB

4B5.6-Sepharose as described in Example 13 and the Friedewald method.

Figure 22: Binding curves of the HRPO-digitonin conjugate of Example 16 to Lp(a)-cholesterol, LDL-cholesterol and VLDL-cholesterol particles.

Figure 23: A typical calibration curve plot of Lp(a)-cholesterol concentration versus absorbance prepared using the method of Example 18.

## DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method for determining the amount of cholesterol associated with a specific lipoprotein, such as LDL-cholesterol, in a sample. A lipoprotein specific binding agent and a sample are mixed and incubated. The amount of cholesterol associated with the lipoprotein of interest present in the sample is then determined from the amount of cholesterol present in the binding-agent-lipoprotein complexes formed in the reaction.

The claimed method utilizes a lipoprotein specific binding agent to form a binding complex with lipoprotein particles in a sample. Following separation of the sample and the binding-agent-lipoprotein complexes, the amount of cholesterol associated with the lipoprotein in the complex is then measured. Preferably, the lipoprotein particles are captured by a lipoprotein specific binding agent directly or indirectly bound to a solid support. This simplifies the separation of the resulting binding-agent-lipoprotein complexes.

Lipoprotein specific binding agents include lipoprotein specific binding proteins, such as monoclonal and polyclonal antibodies and other lipoprotein specific synthetic or recombinant proteins that specifically bind lipoprotein cholesterol particles. For example, an LDL specific binding agent will include LDL specific binding proteins, such as monoclonal and polyclonal antibodies and other LDL specific synthetic and recombinant proteins, that specifically bind

LDL-cholesterol particles. Lipoprotein cholesterol particles are particles composed of a lipoprotein, such as LDL, VLDL, IDL, Lp(a), HDL and the like, containing cholesterol either directly or indirectly bound, coupled (ionically or covalently), absorbed or adsorbed to the lipoprotein particles.

Preferably, the specific lipoprotein-cholesterol particles of interest are separated from other lipoprotein cholesterol particles in the sample before the cholesterol determination is made. For example, LDL-cholesterol particles are selectively separated from HDL, Lp(a), IDL and VLDL-cholesterol particles prior to the measurement of the cholesterol associated with the LDL particles.

The lipoprotein specific binding agent can be attached directly or indirectly to a solid support, for example, by absorption, adsorption, covalent coupling directly to the support or indirectly through another binding agent (such as an anti-antibody antibody), or the like. The type of attachment or binding will typically be dependent upon the material composition of the solid support and the type of lipoprotein specific binding agent used in the assay. For example, nitrocellulose, polystyrene and similar materials possess chemical properties that permit absorption or adsorption of proteins to a solid phase composed of this material. Other materials, such as, latex, nylon and the like contain groups that permit covalent coupling of the lipoprotein specific binding agent to the solid support. Groups, such as, amines and carboxylic acids are coupled through the activation of the carboxylic acid group with, for example, carbodiimide compounds, to form an amide linkage. Other linking methods are well-known in the art particularly for coupling proteins to solid phases and one skilled-in-the-art can easily conceive of a variety of methods for covalently coupling the specific binding agent to the solid support. The solid support can take the form of a variety of materials, for example, the solid support may be in the form of a bead particle, a magnetic particle, a strip or a layered device.

The separation of the binding-agent-lipoprotein complexes from the sample or more specifically from the other lipoprotein particles in the sample can be accomplished in a variety of ways. When the binding agent is coupled to a solid support, the solid support can be removed from the sample or the sample can be removed from the solid support. For example, when the solid support is a microtiter plate or another type of reaction well device, such as the devices described in US Patents 5,075,077 and 4,883,763 and US Patent Application Serial No. 523,629, incorporated herein by reference, the sample can be removed from the wells and the plate washed of any residual sample. When the solid support is a particle, such as a latex or magnetic particle, the solid support can be separated from the sample by filtration through a fiber matrix, such as the devices described in US Patents 4,552,839 and 5,006,309, US Patent Applications Serial Nos. 554,975, 611,235 and 425,651, and Fiore, et al (1988) Clin. Chem. 34/9:1726-1732, all of which are incorporated herein by reference, or by attraction to a magnet followed by removal of the particles or the sample. Alternatively, the binding-agent-lipoprotein complexes can be separated or removed by filtration such as by the Ion Capture Methodology described in co-pending US Patent Application Serial No. 150,278 and US Patent Application Serial No. 375,029, both of which enjoy common ownership and both of which are incorporated herein by reference. These applications describe the use of ion capture separation, in which specific binding members used in an assay are chemically attached to a first charged substance and a porous matrix having bound thereto a second charged substance that binds to the first charged substance. A specific binding pair is formed and separated from the reaction mixture by an electrostatic interaction between the first and second charged substances. The specific binding member is preferably covalently coupled to the first charged substance. Examples of charged substances include anionic and cationic monomers or polymers, such as polymeric acids, e.g.

polyglutamic acid, polyaspartic acid, polyacrylic acid and polyamino acids; proteins and derivative proteins, such as albumin; anionic saccharides, such as heparin or alginic acid; polycations, such as GafQuat™, diethylaminoethyl-dextran and cellulose derivatives such as polymeric quaternary ammonium compounds, such as Celquat™ L-200 and Celquat™ H-100. One skilled in the art is well versed in the separation of solid supports and samples.

The amount of cholesterol in or on a lipoprotein particle can be determined by a variety of methods. For example, lipoprotein cholesterol (such as LDL-cholesterol) can be detected chemically by using the Liebermann-Burchard method or modifications of their method; enzymatically using a cholesterol specific enzyme such as cholesterol oxidase; through the formation of a cholesterol specific binding complex, such as an anti-cholesterol antibody/cholesterol complex; or through the release of the cholesterol from the lipoprotein followed by detecting the amount of cholesterol released using any of the above methods. One skilled-in-the-art may conceive of yet other methods of detection applicable to this method.

For purposes of illustration, an LDL-cholesterol measurement can be made as follows. LDL particles present in a plasma sample are specifically captured by an LDL-specific monoclonal antibody immobilized on a solid support. After separating the solid support from the other unbound plasma lipoproteins, the cholesterol content of the bound LDL particles is estimated by releasing the cholesterol and its esters with a detergent solution. A Standard Cholesterol assay reagent comprising of cholesterol ester hydrolase, cholesterol oxidase and horseradish peroxidase is added. The liberated hydrogen peroxide is then quantitated using a Tinder dye reagent comprising of 4-aminoantipyrine and 3,5-dichloro-2-hydroxybenzenesulfonic acid similar to that described (Sidel et al (1983) *Clin. Chem.* 29:1075-1079). The cholesterol concentration in a given sample is quantitated on the basis of the color generation.

Alternatively, a sandwich immunoassay method for the quantitation of LDL-cholesterol in a plasma sample can be used. This involves the specific capture of the LDL particles in the plasma sample by the LDL-specific antibody immobilized on the solid support followed by quantitation of cholesterol in the captured LDL particles by a cholesterol binding agent which is coupled directly or indirectly to a label. The LDL-cholesterol bound cholesterol binding agent is then quantitated by detection and measurement of the label.

Another alternative is based on an immunochromatographic assay format (such as described in US Patent 4,954,452 and co-owned and copending U.S. Patent Application Serial No. 072,459, incorporated herein by reference), in which the lipoprotein particles in the test sample bind to a labeled cholesterol binding agent. The resulting complexes then travel along a test strip by capillary action. The labeled LDL complexes are then captured by a high affinity anti-LDL specific antibody immobilized on the test strip followed by detection and measurement of the captured labeled LDL complexes. Typically, the test strip is comprised of a porous or bibulous membrane and the result is determined by a visual readout of a detectable signal.

The term "label", as used herein, refers to any substance which can be attached to specific binding agents, such as antibodies, antigens, cholesterol binding agents, lipoprotein specific binding agents and analogs thereof, and which is capable of producing a signal that is detectable by visual or instrumental means. Various suitable labels for use in the present invention can include chromogens, catalysts, fluorescent compounds, chemiluminescent compounds, radioactive elements, colloidal metallic (such as gold), non-metallic (such as selenium) and dye particles (such as the particles disclosed in U.S. Pat. Nos. 4,313,734, 4,954,452 and 4,373,932, incorporated herein by reference), enzymes, enzyme substrates, and organic polymer latex particles (as disclosed in co-owned and copending U.S. Patent Application Serial No. 248,858, filed September 23, 1988, which is incorporated by

reference herein), liposomes or other vesicles containing such signal producing substances, and the like. A large number of enzymes suitable for use as labels are disclosed in U.S. Patent No. 4,275,149, incorporated herein by reference. Such enzymes include phosphatases and peroxidases, such as alkaline phosphatase and horseradish peroxidase which are used in conjunction with enzyme substrates, such as nitro blue tetrazolium, 3,5',5,5'-tetranitrobenzidine, 4-methoxy-l-naphthol, 4-chloro-l-naphthol, 5-bromo-4-chloro-3-indolyl phosphate, chemiluminescent enzyme substrates such as the dioxetanes described in US Patents 4,857,652 (issued August 15, 1989), 4,931,223 (issued June 5, 1990), 4,931,569 (issued June 5, 1990), 4,962,192 (issued October 9, 1990) and 4,978,614 (issued December 18, 1990), incorporated herein by reference, and derivatives and analogs thereof. Fluorescent compounds such as fluorescein, phycobiliprotein, rhodamine and the like, including their derivatives and analogs are suitable for use as labels.

Cholesterol binding agents bind specifically to cholesterol and include digitonin, tomatine, filipin, amphotericin B and specific binding proteins such as polyclonal and monoclonal antibodies and other synthetic and recombinant proteins that specifically bind cholesterol, cholesterol esters and/or the cholesterol associated with lipoprotein particles. A number of cholesterol binding agents are known in the literature. These include saponins such as digitonin (Berezin et al. (1980) Vopr. Med. Khim. 26:843-846; Tsybul's kaya et al. (1986) Bioorg. Khim. 12:1391-1395), tomatine (Schultz and Sanders (1957) Z. Physiol. Chem. 308:122-126; Eskelson et al. (1967) Clin. Chem. 13:468-474), filipin (Boernig et al. (1974) Acta Histochem. 50:110-115; Behnke et al. (1984) Eur. J. Cell Biol. 35:200-205), amphotericin B (Braitburg et al. (1984) J. Infect. Dis. 149:986-997), triterpene glycoside halotoxin A1 and related compounds (Ivanov et al. (1986) Vopr. Med. Khim. 32:132-134). Both monoclonal and polyclonal antibodies to cholesterol are also

known (J. Immunol. (1964) 92:515; Nature (1965) 407; Proc. Natl. Acad. Sci. USA (1988) 85:1902).

Digitonin, tomatine, amphotericin B and antibodies can be used in the quantitation of cholesterol and its esters in lipoprotein particles. Digitonin and tomatine were chemically modified and then conjugated to horseradish peroxidase (HRPO) and alkaline phosphatase (AP). Amphotericin B and anti-cholesterol antibodies were coupled directly to HRPO and AP. All four HRPO and AP conjugates bind to cholesterol and its esters in lipoproteins which are immobilized on a solid phase. The binding affinity of the enzyme conjugates follow the order digitonin > tomatine > anti-cholesterol antibodies > amphotericin B. Because digitonin conjugates and tomatine conjugates bound effectively to the cholesterol components of lipoproteins, these conjugates are preferred in the present invention.

A method of the present invention is illustrated by the following sandwich assay example. The method involves incubating the sample with a solid phase having an LDL specific binding agent, such as the monoclonal antibody 4B5.6, immobilized on a solid phase and the remaining non-specific binding sites of the solid phase blocked, such as with bovine serum albumin or alkali-treated casein. LDL particles are captured by the antibody on the solid phase. Digitonin or tomatine enzyme conjugates are then incubated with the solid phase. The conjugate binds to the cholesterol associated with the LDL particles on the solid phase. The quantity or presence of enzyme bound to the solid phase or the quantity of unbound conjugate remaining after incubation with the solid phase is determined by incubation of enzyme substrate with the solid phase or the solution containing unbound conjugate. The presence of cholesterol associated with the captured LDL particles is then determined from the presence of enzyme associated with the solid phase or a reduction of enzyme activity in the solution containing unbound conjugate as compared with the original conjugate solution added to the solid phase. The quantity of cholesterol associated with the

captured LDL particles is proportional to the quantity of enzyme associated with the solid phase or inversely proportional to the quantity of unbound conjugate. This method is also applicable for any of the other lipoprotein particles or mixtures thereof by substituting the appropriate lipoprotein specific binding agent for the LDL specific binding agent.

A critical aspect of this invention is the selection of the lipoprotein specific binding agents. A lipoprotein specific binding agent preferably must selectively bind to the lipoprotein of interest, but not to other lipoproteins. For example, an LDL specific binding agent preferably binds only to LDL and not to other lipoproteins, such as HDL, VLDL, IDL and Lp(a). In addition, when the cholesterol binding agent is an antibody, the lipoprotein specific binding agent and the cholesterol binding antibody must be compatable such that neither binding agent interferes with the binding of the other agent to the lipoprotein particle and cholesterol associated with the particle. The preferred lipoprotein specific binding agent is an antibody which binds specifically to the lipoprotein of interest. The antibody is preferably a monoclonal antibody. Monoclonal antibodies are preferable because production quantities of antibody are readily available and such antibodies generally improve the lot-to-lot reproducibility and consistency in the assay results.

The term antibody is also meant to include both intact molecules as well as fragments thereof, such as, for example, Fab and $F(ab')_2$, which are capable of binding antigen. Fab and $F(ab')_2$ fragments lack the Fc fragment of intact antibody and may have less non-specific binding than an intact antibody (Wahl, et al., J. Nucl. Med. 24:316-325, 1983). Such fragments also may be used for the detection and quantitation of lipoprotein cholesterol particles according to the methods disclosed herein in the same manner as intact antibodies. Such fragments are well known in the art and are typically produced by enzymatic degradation of an antibody, such as with pepsin, papain or trypsin. Alternatively, antibodies and

antibody fragments can be prepared using recombinant antibody methods such as those described in US Patent Applications Serial Nos. 513957, 693249, 789619, 776391, 799770, 799772, and 809083, incorporated herein by reference, wherein antibodies or antibody fragments are produced from the RNA of an antibody producing B-cell from an immunized animal, such as a rat or mouse, using known recombinant techniques.

Lipoprotein specific binding agents according to the present invention also include bacteriophage described in US Patent 4,797,363, incorporated herein by reference. Bacteriophage tail or head segments are capable of selectively binding antigens. By mutation and selection processes, bacteriophage having the necessary binding characteristics to selectively bind lipoprotein cholesterol particles can be obtained.

Lipoprotein specific binding agents according to the present invention also include nucleic acid sequences, such as DNA and RNA, which selectively bind to lipoprotein cholesterol particles. A library of nucleic acid sequences are tested for the desired binding characteristics and the sequences that are specific for lipoprotein cholesterol particles are isolated and replicated. Weintraub, et al., WO 92/05285, and Gold, et al., WO 91/19813, both incorporated herein by reference, disclose methods for the preparation of DNA and RNA sequences which are antigen specific.

Preferably, the lipoprotein specific binding agent is selective for only one lipoprotein, but some recognition of or binding to other lipoproteins can be tolerated. For example, an antibody selected for its ability to bind only to LDL particles present in a sample can minimally capture other lipoproteins and still be utilized in this invention. More specifically, the present invention can tolerate an LDL specific monoclonal antibody that does not cross-react with Lp(a) or HDL, but does cross-react with VLDL up to 20% and with IDL up to 20%. In addition, the LDL specific monoclonal antibody should not

cross-react with other lipoprotein and non-lipoprotein materials present in a sample.

The measurement of a specific lipoprotein cholesterol level can also be accomplished indirectly by removing all the other lipoproteins from the sample. The selective binding agents of a group of selected lipoproteins can be used to remove these lipoproteins from a sample, leaving behind substantially only one lipoprotein in the sample. Measurement of the cholesterol in the sample after this group of lipoproteins have been removed gives an indication of the amount of cholesterol present in the remaining lipoprotein. For example, selectively removing HDL, VLDL, IDL and Lp(a) will essentially leave behind LDL in the sample. Measurement of the cholesterol in the remainder of the sample gives an indication of the LDL-cholesterol present in the sample. The cholesterol levels associated with the other lipoproteins could be measured by simply changing the group of selected lipoproteins removed from the sample. Moreover, lipoprotein specific binding agents, such as antibodies, that are not selective for only one lipoprotein, such as an antibody that binds to both VLDL and LDL but not Lp(a), can be used to remove the antibody cross-reacting lipoproteins (VLDL and LDL) in the measurement of cholesterol associated with a non-cross reacting lipoprotein (Lp(a)) using this indirect method.

This indirect approach can also improve the efficacy of lipoprotein specific binding agents, used in the direct measurement of a specific lipoprotein, that are not sufficiently selective for the lipoprotein of interest. By removing an antibody cross-reacting specific lipoprotein from the sample prior to specifically measuring the lipoprotein cholesterol of interest, the effect of such cross-reactivity is eliminated.

Moreover, the sequential removal and measurement of specific lipoprotein cholesterol levels from the same aliquot of sample permits the use of less selective lipoprotein selective binding agents in the measurement of lipoprotein cholesterol levels later in the sequence. For example, an antibody that binds to both VLDL and LDL could be used to

selectively capture VLDL if the LDL present in the sample had previously been removed.

The present invention has the capability of quantitating the amount of cholesterol associated with lipoproteins. This advancement in technology will more clearly define the correlation between lipoprotein cholesterol levels and CHD. For example, by specifically detecting LDL-cholesterol and not the cholesterol associated with other lipoproteins present in a plasma sample, the present invention improves the precision, accuracy and reproducibility of LDL-cholesterol measurement of a sample and thereby a better indication of the association of the LDL-cholesterol level with CHD. Thus, the dietary or therapeutic drug treatment of a patient can now be more carefully monitored for improved results in lowering the risk of CHD in the patient. Moreover, the simplicity of performing LDL-cholesterol measurements will also be improved. Determination of LDL-cholesterol levels by multiple measurements of various lipoprotein cholesterol levels will no longer be necessary.

The following examples are illustrative of the invention and are in no way to be interpreted as limiting the scope of the invention as defined in the claims. It will be appreciated that one skilled-in-the-art can conceive of many other devices and methods for use of which the present inventive concepts can be applied.

LDL-CHOLESTEROL SPECIFIC ASSAY

1. SOURCES OF THE MONOCLONAL ANTIBODIES

Monoclonal antibodies (MABs) were procured from five different laboratories. SPL4A5, 8A2.1, 8B3.5, and 8A6.6 ($IgG_1$), and 4B5.6 ($IgG_{2b}$) were obtained from Alexander Karu, University of California at Berkeley as ascites precipitated by 50% ammonium sulfate and dialyzed against TRIS buffer pH 7.2. The MAB SPL4A5 is described in U.S. Patent 4,619,895 for use in the diagnosis of patients suffering from Type IV

hypertriglyceridemia. The blood of such patients contains LDL particles of size between 215-230 Å which is not present in normal individuals. The antigenic epitope of these LDL particles which is recognized by the MAB SPL4A5 was not disclosed. The MABs 8A2.1, 8B3.5, 8A6.6 and 4B5.6 were described in LaBelle, et al. (1990) Clin. Chimica. Acta. 191:153-160. These four MABs reportedly reacted with LDL and apo B, but not with HDL. The antigenic epitopes of the LDL and apo B which were recognized by the four MABs were determined by Western blot using apo B fragments cleaved from apo B by thrombin. The MABs 8A2.1, 8A6.6 and 8B3.5 recognized thrombolytic fragment T3 and the MAB 4B5.6 recognized only thrombolytic fragment T2. Prior to use in the experiments herein, the MABs were purified on a Protein A Sepharose column as described in Example 1 (*infra*). The MABs SPL4A5, 8A2.1, 8A6.6 and 8B3.5 were eluted from the column with pH 6.0 citrate buffer and the MAB 4B5.6 was eluted from the column with pH 4.0 citrate buffer.

MABs BI9, B18, B06, B05, B04 and B02 were obtained from Jean-Charles Fruchart, SERLIA Institut Pasteur, Lille Celdex, France, as purified IgG fractions.

MABs 457C4DI, 465C3DI, 464BIB3, and 464BIB6 were obtained from Gustav Schonfeld, Washington University Medical Center, St. Louis, MO. These MABs are all $IgG_1$ and were described in Tikkanen, et al. (1982) J. Lipid Res. 23:1032-1038; and Tikkanen, et al. (1983) J. Lipid Res. 24:1494-1499. The MABs differed in reactivity toward LDL obtained from different individuals and were suggested to recognize different antigenic epitopes of LDL. The MABs were supplied in ascites fluid and were purified on Protein A Sepharose column as described in Example 1 (*infra*). All four antibodies were eluted from the column with citrate buffer at pH 6.0.

MABs apo $B_{sol}16$ (MB16), 2D8 and IDI ($IgG_1$), and 5EII ($IgG_{2a}$) were obtained from Yves Marcel, Clinical Research Institute of Montreal, Canada. The MABs were characterized and the antigenic epitopes of apo B recognized by these MABs was determined using apo B fragments cleaved from apo B by

thrombin, synthetic oligopeptides and recombinant proteins (Marcel, et al. (1987) Mol. Immunol. 24:435-447; Marcel, et al. Arteriosclerosis (1987) 7:166-175; Chen, et al. (1988) Eur. Biochem. 175:111-118; and Knott, et al. (1986) Nature 323:734-738). MAB MB16 recognized the peptide residues 4154-4189 of thrombolytic fragment T2. MAB IDI recognized the peptide residues 1-1297 of thrombolytic fragment T4. MAB 2D8 recognized the peptide residues 1297-2177 of thrombolytic fragment T3. MAB 5EII recognized the peptide residues 2488-3636 in the T3/T2 region of LDL (B,E) receptors. All four MABs were reported to react with LDL and solubilized apo B. Regarding their reactivity with VLDL, MABs IDI, 2D8 and 5EII required the presence of lipids in order to react with VLDL, whereas MAB MB16's reactivity was found to be lipid independent. The MABs were obtained as 50% ammonium sulfate precipitates of the ascites fluid. Prior to use herein, the MABs were purified on Protein A Sepharose columns as described in Example 1 (*infra*). MAB 5EII was eluted from the column with pH 5 citrate buffer and the others were eluted from the column with pH 6 citrate buffer.

Three purified anti-LDL monoclonal antibodies A016-08, A016-09, and A016-10 were obtained from Medix Biotech, Foster City, CA. All three purified anti-LDL MABs were characterized in terms of their cross-reactivities with other lipoproteins in order to select LDL-specific antibodies. The selected antibodies bound to LDL particles of blood samples from normal individuals and from individuals with high triglycerides blood levels (300-400 mg/dL), did not bind to Lp(a) or HDL, and reacted with VLDL and IDL at less than about 10% of the LDL reactivity. Suitable antibodies for use in the present invention bind to (1) Lp(a) preferably at less than about 5% of the LDL reactivity and more preferably at less than about 2% of the LDL reactivity; (2) HDL preferably at less than about 2% of the LDL reactivity and more preferably at less than about 1% of the LDL reactivity; and (3) VLDL and IDL, preferably at less than about 20% of the LDL reactivity and more preferably at less than about 10% of the LDL reactivity.

2. EVALUATION OF THE MONOCLONAL ANTIBODIES

The following methods were used to evaluate the antibodies.

a. Direct ELISA using lipoprotein coated microtiter plates

Lipoprotein fractions (LDL, HDL, VLDL, IDL and Lp(a)) purified by ultracentrifugation (see Example 2, *infra*) were coated on separate wells of a Maxisorb Nunc Immuno Plate as follows: one hundred microliters (100 µL) of each lipoprotein fraction at a lipoprotein-cholesterol concentration of about 1 µg/ml in 20 mM phosphate buffered saline at pH 7.0 (PBS) was dispensed into separated wells of the microtiter plate, the plate was incubated at 37°C for one hour, and the plate was then washed five times with PBS containing 0.05% (v/v) Tween 20 (PBS-Tween 20). The non-specific binding sites were blocked with 200 µL of 10% (v/v) fetal bovine serum (FBS) in PBS at 37°C for one hour and then were washed five times with PBS-Tween 20. Each MAB was diluted in 3% (v/v) FBS in PBS to a final antibody concentration of about 2 µg/ml and the diluted MAB solutions were then serially diluted on the plates. After incubation at 37°C for one-half hour, the plate was washed five times with PBS-Tween 20. Thereafter, one hundred microliters (100 µL) of horseradish peroxidase (HRPO) labeled goat anti-mouse IgG (obtained from Kirkegaard and Perry Laboratories, MD), diluted in 3% FBS in PBS to a final concentration of about 1.25 µg/mL, were added to each reaction well and the plate was incubated at 37°C for one-half hour. The plate was then washed eight times with PBS-Tween 20. One hundred microliters (100 µL) of freshly prepared HRPO substrate solution, containing one o-phenylenediamine (OPD) tablet per five milliliters (5 mL) of citrate buffer at pH 6 (both available from Abbott Laboratories, IL), were added to each well. The color reaction was stopped after five minutes by adding 100 µL of 1N $H_2SO_4$ to the reaction wells. An

absorbance reading of each reaction well was then obtained with a Bio-Tek microplate reader at 490 nm. Typical binding curves for each lipoprotein tested with MAB MB16 are shown in Figure 1. A summary of test results are presented in Table 1 which shows the binding efficiencies of the lipoproteins relative to LDL at an antibody concentration of about 1 µg/mL. MABs 4B5.6 and A016-08 did not show any binding with LDL even at an antibody concentration of about 2 µg/mL. MABs SPL4A5, 8A2.1 and 465C3DI produced absorbance readings of

TABLE 1

BINDING OF MABs TO LIPOPROTEINS ON SOLID PHASE

| MAB* | LDL | VLDL | IDL | Lp(a) | HDL |
|---|---|---|---|---|---|
| B19 | 100 | 40 | 46 | 2 | 0 |
| B18 | 100 | 20 | 62 | 3 | 0 |
| B06 | 100 | 18 | 59 | 2 | 0 |
| B05 | 100 | 57 | 80 | 1 | 0 |
| B04 | 100 | 34 | 72 | 0 | 0 |
| B02 | 100 | 9 | 77 | 0 | 0 |
| 4B5.6 | 0 | 0 | 0 | 0 | 0 |
| SPL4A5 | 100 | 42 | 84 | 0 | 0 |
| 8A2.1 | 100 | 32 | 90 | 0 | 0 |
| 8B3.5 | 100 | 26 | 9 | 0 | 0 |
| 8A6.6 | 100 | 15 | 69 | 0 | 0 |
| 465C3D1 | 100 | 0 | 0 | 0 | 0 |
| 457C4D1 | 100 | 28 | 19 | 0 | 0 |
| 464B1B3 | 100 | 42 | 32 | 0 | 0 |
| 464B1B6 | 100 | 41 | 26 | 0 | 0 |
| MB16 | 100 | 23 | 43 | 16 | 7 |
| 2D8 | 100 | 38 | 60 | 12 | 5 |
| 1D1 | 100 | 22 | 36 | 13 | 0 |
| 5E11 | 100 | 34 | 51 | 18 | 4 |
| A016-08 | 100 | 0 | 0 | 0 | 0 |
| A016-09 | 100 | 47 | 67 | 20 | 0 |
| A016-10 | 100 | 54 | 78 | 27 | 4 |

* At 1 µg/mL antibody concentration.

about 0.2 at 490 nm. The low binding (affinity) of these MABs may possibly be due to unfavorable orientation of the lipoproteins on the solid phase which could interfere with the accessibility of the antigenic epitopes by the antibodies resulting in little or no binding by the antibodies. These MABs would thus be lipoprotein conformation-dependent.

b. Specificity of the antibodies for LDL using lipoprotein coated microtiter plates in competitive assays

The specificities of the MABs were determined by competitive binding of the MABs to the other lipoproteins in microplate wells coated with LDL. The LDL-coated plates were prepared as described previously (see section 2a above). Each MAB was diluted with 3% (v/v) FBS in PBS to a concentration that was two times the MAB concentration at 50% LDL-binding as determined from the binding curves prepared in section 2a above. Examples of such curves are shown in Figure 1. Purified lipoprotein samples were diluted in PBS starting at the following cholesterol concentrations: LDL-cholesterol concentration of 45 mg/dL; VLDL-cholesterol concentration of 45 mg/dL; IDL-cholesterol concentration of 22.5 mg/dL; HDL-cholesterol concentration of 22.5 mg/dL; and Lp(a) at a total mass of Lp(a) of 22.5 mg/dL. Fifty microliters (50 µL) of each lipoprotein solution were then serially diluted with PBS in reaction wells blocked by 10% (v/v) FBS in PBS. To each of these wells were added 50 µL of the diluted MAB solutions. The MAB-lipoprotein mixtures were incubated at room temperature for one-half hour on a rotator at 100 rpm. The contents from each well were then transferred to LDL-coated reaction wells and the plates were incubated at 37°C for one-half hour. The amount of MAB bound to the LDL-coated reaction wells were measured according to the method described in section 2a above. Typical competitive binding curves are shown in Figure 2. A summary of the test results are presented in Table 2. The cross-reactivities were

determined at 50% inhibition of binding by a competing lipoprotein using the following equation:

$$\text{cross-reactivity (\%)} = \frac{\text{amount needed by LDL}}{\text{amount needed by competitor}} \times 100$$

MAB B18 did not show any inhibition of binding even by LDL. The results also indicate extensive binding of all MABs, particularly with IDL. This is in contrast to the binding curves obtained in section 2a above. For example, MB16 in Figure 1 shows much less affinity towards VLDL and IDL, whereas MB16 in Figure 2 shows almost equal affinity for both LDL and IDL. This indicates that the affinity of the MABs toward lipoproteins differs depending on whether the reaction

TABLE 2

COMPETITIVE BINDING OF MABs BY ELISA

| MAB | % Cross-Reactivity with LDL | | | |
|---|---|---|---|---|
| | VLDL | IDL | Lp(a) | HDL |
| B19 | 42 | 53 | 1 | 0 |
| B18 | NO INHIBITION WITH LDL OR OTHER LIPOPROTEINS | | | |
| B06 | 37 | 52 | 7 | 0 |
| B05 | 34 | 82 | 5 | 0 |
| B04 | 39 | 59 | 6 | 0 |
| 8B3.5 | 35 | 100 | 0 | 0 |
| 8A6.6 | 50 | 100 | 0 | 0 |
| 457C4D1 | 50 | 100 | 0 | 0 |
| 464B1B3 | 47 | 94 | 5 | 0 |
| 464B1B6 | 29 | 81 | 15 | 0 |
| MB16 | 100 | 105 | 21 | 10 |
| 2D8 | 56 | 105 | 50 | 48 |
| 1D1 | 140 | 100 | 37 | 30 |
| 5E11 | 41 | 41 | 21 | 21 |
| A016-09 | 95 | 100 | 22 | 22 |
| A016-10 | 100 | 55 | 29 | 25 |

is done with a solid phase (Figure I) or in a fluid phase (Figure 2). Therefore, any published conclusions relating to the reactivities of these MABs using only one of these methods is suspect and unreliable. Thus, we developed an assay where lipoprotein specific antibodies are immobilized on the solid phase in order to capture specific lipoproteins to avoid the inconsistencies observed in the above methods.

c. Direct ELISA using antibody coated microtiter plates

MABs were coated onto the reaction wells of microtiter plates as follows. MABs were diluted in PBS as follows: SPL4A5 (20 µg/mL), 8A2.1 (20 µg/mL), 8A6.6 (2 µg/mL), 4B5.6 (3 µg/mL), 8B3.5 (5 µg/mL), 465C3D1 (2 µg/mL), 457C4DI (2 µg/mL), 464B1B3 (1.5 µg/ml, 464BIB6 (1.5 µg/mL), B19 (2 µg/mL), B18 (2.5 µg/mL), B06 (10 µg/mL), B04 (10 µg/mL), B02 (4 µg/mL), MB16 (5 µg/mL), IDI (5 µg/mL), 2D8 (5 µg/mL), 5EII (5 µg/mL), A016-08 (10 µg/mL), A016-09 (10 µg/mL) and A016-10 (10 µg/mL). One hundred microliters (100 µL) of each MAB solution were dispensed into separate reaction wells and incubated at room temperature on a rotator at 100 rpm for two hours. The plates were then washed five times with PBS-Tween 20 and blocked with 200 µL of 10% FBS in PBS by incubation at 37°C for one hour. The plates were then washed five times with PBS-Tween 20.

Each MAB plate was then serially diluted with LDL in PBS, starting with an LDL-cholesterol concentration of 10 mg/dL cholesterol, so that each well contained a total of 100 µL of solution. After incubation at 37°C for one-half hour, the plates were washed five times with PBS-Tween 20. One hundred microliters (100 µL) of 0.6 µg/ml MAB B06-HRPO conjµgate (prepared according to Example 3 and Figure 3) in 3% FBS in PBS were added to each well and incubated at 37°C for one-half hour. HRPO substrate was added and the absorbance measured as described in section 2a above. MABs 4B5.6, SPL4A5, 8A2.1, 465C3D1 and A016-08, which showed minimum binding with the LDL-immobilized ELISA plate even

at 2 µg/mL antibody concentration, provided excellent binding of LDL when the MABs were immobilized on the plate. Figure 4 shows typical binding curves for 4B5.6, SPL4A5, 8A2.1, and 465C3D1. Based on these results, it is likely that the binding affinity of the MABs are dependent on the orientation of the lipoprotein particles, i.e. the MABs are LDL conformation dependent.

d. Competitive RIA on MAB Plate

In order to obtain additional data relating to the specificity of the MABs, competitive radioimmunoassays (RIA) were performed as follows. Iodine-125 labeled LDL (125I-LDL) was prepared enzymatically using immobilized lactoperoxidase and glucose oxidase (Enzymobeads, Bio-Rad) according to the standard procedure described in Tsao, et al. (1987) *J. Biol. Chem.* 257:15222-15228. The specific activity ranged between 1.87-2.60 µCi/µg. The 125I-LDL sample was stored at 4°C in 0.1 M TRIS-saline pH 7.5 containing 10 mg/ml lipid-free bovine serum albumin (BSA) (Armour CRG-7) and was used within 18 days after preparation. In the first series of experiments, the binding of 125I-LDL to each MAB immobilized on Immulon 2 Removawell ELISA strips was evaluated. Typical binding curves of 125I-LDL using MABs 2D8, 1D1 and MB16 are shown in Figure 5. The Removawell plates were prepared as follows. The concentrations of the MABs used in the preparation of the Removawell plates were exactly the same as described in section 2c above. One hundred microliters (100 µL) of each MAB solution in PBS were added to each well and incubated at 37°C for one hour. The plates were washed five times with PBS and then blocked with 200 µL of 10% FBS in PBS. The plates were then washed five times with PBS. The lipoproteins were diluted in PBS to the following lipoprotein-cholesterol concentrations: LDL-cholesterol, VLDL-cholesterol and HDL-cholesterol concentrations of 20 mg/dL; IDL-cholesterol concentration of 10 mg/dL; and Lp(a) at a Lp(a) total mass of 20 µg/dL. Fifty

microliters (50μL) of each lipoprotein solution were serially diluted in PBS in microtiter reaction wells blocked by 10% FBS in PBS. Fifty microliters (50μL) of 125I-LDL (100,000 cpm) diluted in 3% (w/v) BSA in PBS were added to each well. The contents from each well were transferred completely to respective MAB Removawell plates. The plates were incubated at room temperature on a rotator at 100 rpm for 20 hours. The plates were then washed eight times with PBS. Each well was then transferred to Falcon polystyrene tubes (12 x 75 mm) and the bound radioactivity was counted on an APEX automatic Y-counter. The background was subtracted to calculate the net binding. The competitive displacement curves for 4B5.6 are shown in Figure 6. The cross-

TABLE 3

COMPETITIVE BINDING OF MABs BY RIA

| MAB | % Cross-Reactivity with LDL | | | |
|---|---|---|---|---|
| | VLDL | IDL | Lp(a) | HDL |
| B19 | 26 | 63 | 33 | 0 |
| B02 | 80 | 58 | 9 | 0 |
| 8B3.5 | 42 | 61 | 44 | 0 |
| 8A6.6 | 100 | 12 | <2 | 0 |
| 8A2.1 | 7 | 7 | <2 | 0 |
| 465C3D1 | 15 | 50 | 8 | 0 |
| 457C4D1 | 83 | 69 | 4 | 0 |
| 464B1B3 | 153 | 345 | 92 | 0 |
| 464B1B6 | 73 | 393 | 110 | 0 |
| MB16 | 19 | 21 | 6 | 0 |
| 2D8 | 50 | 27 | 12 | 6 |
| 1D1 | 42 | 63 | 14 | 0 |
| 5E11 | 42 | 27 | 12 | 6 |
| 4B5.6 | 7 | 18 | 2 | 0 |
| SPL4A5 | 17 | 7 | <1 | 0 |
| A016-08 | 64 | 33 | 13 | 0 |
| A016-09 | 56 | 43 | 8 | 0 |
| A016-10 | 69 | 56 | 13 | 0 |

reactivities were determined for each MAB at 50% inhibition of binding using the equation in section 2b above. A summary of results are shown in Table 3.

The evaluation of the monoclonal antibodies described above indicates that at least two types of antigenic epitopes exist: one dependent mostly on the peptide sequence of the apo B fragments and the other dependent on the conformation dictated by the presence of associated lipids in the lipoproteins and also on the sizes of the particles. The results are summarized in Figure 7A. The antigenic epitopes of apo B fragments formed by thrombin which are known are shown in Figure 7B and the antigenic epitopes of apo B are known to bind the following MABs MAB 1D1 binds to the T4 fragment 1-1297. MAB 2D8 binds to the T3 fragment 1297-2177. MABs 8A2.1, 8A6.6 and 8B3.5 bind to the T3 fragment 1297-3249. MAB 5EII binds to the T3/T2 fragment 2488-3636. MAB MB47 binds to the LDL (B,E) receptor binding fragment 3350-3506 (Weisgraber et al (1988) Proc. Natl. Acad. Sci., USA 85:9758-9762). We found that all these MABs are highly cross-reactive with other apo B containing lipoproteins, such as VLDL and IDL. The MAB SPL4A5, for which the binding epitope of apo B is not known, appears to have higher affinity for LDL with smaller particle size as outlined in the U.S. Patent 4,619,895. This MAB was found to be less cross-reactive to VLDL (17%) and IDL (7%). MAB MB16 is known bind to the T2 fragment 4154-4189. We have determined that MAB MB16 had minimal cross-reactive with VLDL (19%) and IDL (20%). MAB 4B5.6 is known to bind to the T2 fragment 3249-4536. We determined that MAB 4B5.6 had minimal cross-reactivity with VLDL (7%) and IDL (18%). Thus, MABs, which are specific for the apo B T2 fragment or sub-fragments thereof, will bind to the apo B T2 fragment and LDL, but will have low (less than 20%) cross-reactivity with VLDL, IDL and Lp(a). Such MABs would be useful in selectively binding LDL in the presence of other lipoproteins. MABs, which are specific for the apo B T3 fragment or sub-fragments thereof, can also selectively bind to LDL, with low (less than 20%) cross-reactivity with VLDL, IDL and Lp(a), as illustrated

by MAB 8A2.1 in Table 3. However, as will be shown later, MAB 8A2.1's LDL selectivity may be due to particle size because MAB 8A2.1 failed to bind to LDL particles from every plasma sample tested. On the other hand, MABs specific for apo B fragments T4, T3/T2 and sub-fragments thereof will produce MABs which are cross-reactive with other lipoproteins.

Therefore, it is possible to generate LDL-specific MABs using specific peptide fragments of the apo B T2 fragment and possibly also the apo B T3 fragment. Figure 8 shows some typical peptide fragments of apo B T2 fragment useful in generating LDL-specific MABs.

3. IMMOBILIZATION OF MONOCLONAL ANTIBODY TO SOLID PHASE

It was our intention to selectively capture LDL particles on the antibody coated solid phase and assay for the cholesterol in the bound LDL. Cholesterol or other lipids associated with lipoproteins are very hydrophobic. Therefore, it is desirable to use solid phases in an assay for cholesterol which are hydrophilic. Moreover, the solid phase must have high binding capacity and should be non-porous to avoid preferential inclusion of lipoproteins in the porous solid phase. Also, since antibody is to be immobilized on the solid phase, the activity and the orientation of the immobilized antibody must be substantially preserved. We selected CNBr-activated Sepharose 4B (Pharmacia LKB), carbolink hydrazide agarose beads (Pierce Chemicals), and Sulfolink coupling agarose beads (Pierce Chemicals) to demonstrate the feasibility of an LDL specific cholesterol assay. However, any other hydrophilic solid phase, such as Trisacryl (IBF), HEMA-epoxy Bio Gel, HEMA vinylsulfone Bio Gel (Altech Associates), glycosylated silica gel or control porous glass, hydrophilic latex beads, other cellulosic materials etc. can also be used. Examples 4, 5, and 6 show the methods used in the covalent attachment of the monoclonals antibody to CNBr-activated Sepharose 4B, carbolink hydrazide gel and sulfolink gel, respectively.

4. EVALUATION OF ANTIBODY IMMOBILIZED SOLID PHASES

The antibody immobilized solid phases were evaluated in terms of their binding efficiencies by incubating the solid phases with purified LDL fractions from normal subjects and then determining the amount of LDL bound by measuring the amount of cholesterol in the bound solid phases. The cholesterol assay was performed using the reagents as described in Example 7. A typical cholesterol standard curve is shown in Figure 9.

The protocol for the lipoprotein capture assays is described in Example 8. The efficiency of LDL capture on a Sepharose 4B matrix having MAB SPL4A5 bound thereto is shown in Table 4. The result shows that 90% or more LDL particles are being captured on the antibody matrix. Using the same amount of MABs 4B5.6, 8A2.1, and MB16 on CNBr-activated Sepharose 4B, the LDL capture efficiencies were between 92%-98% for LDL having LDL-cholesterol concentrations ranging from about 6 mg/dL to about 24 mg/dL.

In order to investigate whether the other two antibody-matrices, namely hydrazide gel and sulfolink gel, are better than the CNBr-activated Sepharose 4B matrix, a comparative study was undertaken using approximately the same amount of

TABLE 4

EFFICIENCY OF LDL CAPTURE ON SPL4A5-SEPHAROSE

| MAB Matrix* (µL) | LDL** (mg/dL***) | % LDL Captured |
|---|---|---|
| 50 | 6 | 72 |
| 50 | 12 | 83 |
| 100 | 6 | 90 |
| 100 | 12 | 92 |

* MAB Matrix concentration = 2.8 µg/µL.

** 100 µL sample size.

*** LDL concentration is the amount of LDL having the listed LDL-cholesterol concentration.

TABLE 5
LDL CAPTURE EFFICIENCY OF SPL4A5 MATRICES

| MATRIX (100 μL) | AMOUNT OF MAB (μg) | LDL* (mg/dL**) | % LDL Captured |
|---|---|---|---|
| Sepharose 4B | 143 | 6 | 77 |
| | 143 | 12 | 78 |
| | 143 | 24 | 81 |
| Hydrazide | 133 | 6 | 55 |
| | 133 | 12 | 66 |
| | 133 | 24 | 70 |
| Sulfhydryl | 117 | 6 | 60 |
| | 117 | 12 | 66 |
| | 117 | 24 | 66 |

* 100 μL sample size.

** LDL concentration is the amount of LDL having the listed LDL-cholesterol concentration.

antibody on each gel matrix. The results of this study is shown in Table 5. Since the efficiency of LDL-capture in this experiment was lower than in Table 4, the study still clearly demonstrates that the hydrazide gel and the sulfolink gel are not superior to Sepharose 4B. Therefore, Sepharose 4B was selected to demonstrate the new LDL-immunocapture assay. In all future experiments described herein, 100 μL of MAB-Sepharose 4B at an MAB (SPL4A5, 8A2.1, 4B5.6, or MB16) concentration of 2.8 μg/μL were used.

We have shown in the competitive RIA (see Table 3) that the MABs SPL4A5, 8A2.1 and 4B5.6 have weak cross-reactivity with VLDL. Since it is important to know the effect of VLDL present in the plasma along with other lipoproteins in the immunocapture assay, a normal plasma sample with known LDL-cholesterol concentration was used. The plasma was spiked with different amounts of purified VLDL from the same individual and the spiked samples were assayed for the LDL-cholesterol concentration using the present assay method. The results are shown in Table 6. All three MABs provide

similar LDL-cholesterol concentration with increasing VLDL concentration. Also, the measured LDL-cholesterol concentrations increased with increasing VLDL concentrations and the LDL-cholesterol measurements were particularly affected when the VLDL-cholesterol concentration exceed 50% of the LDL-cholesterol concentration. The concentration of VLDL-cholesterol in plasma in most plasma samples is usually less than 50% of the LDL-cholesterol concentration except in a few rare pathological states (such as type III and type IV hypertriglycerimic patients). Generally, an LDL-cholesterol measurement can be affected by VLDL-cholesterol up to ±10% without affecting the clinical significance of the LDL-cholesterol measurement.

This experiment was done with one normal plasma sample and since LDL particle sizes and compositions vary between individuals, the LDL-immunocapture assay was evaluated with a large number of individuals with varied lipoprotein profiles.

TABLE 6

EFFECT OF VLDL ON LDL-CHOLESTEROL MEASUREMENT

| LDL-Chol. (mg/dL) | Spiked VLDL-Chol. (mg/dL) | % VLDL In LDL | LDL -Chol. (mg/dL) using MAB-Sepharose | | |
|---|---|---|---|---|---|
| | | | SPL4A5 | 8A2.1 | 4B5.6 |
| 122 | 18 | 15 | 118 | 120 | 115 |
| 122 | 36 | 30 | 126 | 123 | 126 |
| 122 | 54 | 44 | 130 | 136 | 126 |
| 122 | 90 | 74 | 156 | 161 | 156 |
| 122 | 122 | 100 | 181 | 187 | 183 |

5. LDL-IMMUNOCAPTURE ASSAY

The protocol for the LDL-immunocapture assay is described in Example 9. The LDL-cholesterol concentrations obtained were correlated with the reference methods

ultracentrifugation (ß-quantitation) and Friedewald calculation. The ultracentrifugation method for ß-quantitation is described in Example 10. The Friedewald calculation method for quantitation is described in Example 11.

SPL4A5-Sepharose 4B Matrix: Thirty-four subjects were used in this study (See Table 7 for lipid profiles). The LDL-cholesterol concentrations determined by the two reference methods and the immunocapture assay are presented in Table 8. The correlations between the immunocapture assay and the two reference methods are shown in Figure 10 (A and B). The results indicated that several samples (such as Nos. 15-19) had much lower LDL-cholesterol concentrations as measured by MAB SPL4A5-Sepharose as compared to both reference methods. This suggests that the MAB SPL4A5 alone cannot capture all LDL particles of heterogeneous sizes present in these samples.

TABLE 7

LIPID PROFILES OF PLASMA SAMPLES

| Sampl. No. | Total Chol. mg/dL | HDL-Chol. mg/dL | TRIG[1] mg/dL | F.E.[2] LDL-Chol. mg/dL | U.C.[3] LDL-Chol. mg/dL | VLDL-Chol.[4] mg/dL | Lp(a)-Chol.[5] mg/dL |
|---|---|---|---|---|---|---|---|
| 1 | 138 | 22 | 83 | 99 | 97 | 21 | 0.5 |
| 2 | 181 | 28 | 333 | 86 | 86 | 63 | 1.1 |
| 3 | 128 | 47 | 102 | 61 | 61 | 14 | 1.8 |
| 4 | 119 | 51 | 32 | 62 | 62 | 10 | 15.6 |
| 5 | 104 | 37 | 48 | 57 | 64 | 6 | 15.4 |
| 6 | 183 | 42 | 98 | 121 | 124 | 18 | 19.3 |
| 7 | 187 | 27 | 194 | 121 | 122 | 39 | 18.8 |
| 8 | 158 | 39 | 78 | 103 | 93 | 26 | 1.0 |
| 9 | 128 | 48 | 106 | 59 | 73 | 7 | 7.1 |
| 10 | 116 | 38 | 41 | 70 | 75 | 3 | 2.0 |
| 11 | 157 | 39 | 191 | 80 | 92 | 26 | 4.7 |
| 12 | 117 | 28 | 51 | 79 | 82 | 7 | 5.3 |
| 13 | 142 | 68 | 37 | 66 | 62 | 15 | 7.6 |
| 14 | 179 | 41 | 127 | 113 | 103 | 35 | 5.8 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 15 | 150 | 51 | 85 | 82 | 88 | 10 | 2.1 |
| 16 | 122 | 33 | 50 | 79 | 81 | 18 | 10.0 |
| 17 | 184 | 45 | 132 | 113 | 100 | 38 | 2.5 |
| 18 | 161 | 77 | 94 | 65 | 68 | 28 | 1.2 |
| 19 | 115 | 23 | 77 | 76 | 84 | 8 | 4.7 |
| 20 | 127 | 65 | 117 | 39 | 35 | 34 | 1.2 |
| 21 | 141 | 31 | 171 | 76 | 95 | 15 | 4.8 |
| 22 | 172 | 45 | 70 | 113 | 121 | 16 | 8.6 |
| 23 | 154 | 78 | 68 | 51 | 50 | 22 | 2.3 |
| 24 | 119 | 33 | 51 | 76 | 78 | 8 | 3.1 |
| 25 | 135 | 41 | 114 | 71 | 86 | 8 | 3.1 |
| 26 | 135 | 53 | 53 | 70 | 60 | 22 | 8.5 |
| 27 | 169 | 47 | 235 | 75 | 83 | 39 | 1.4 |
| 28 | 127 | 56 | 83 | 54 | 60 | 11 | 2.8 |
| 29 | 112 | 48 | 49 | 55 | 37 | 17 | 13.0 |
| 30 | 166 | 54 | 35 | 105 | 106 | 7 | 4.1 |
| 31 | 160 | 53 | 145 | 78 | 71 | 36 | 0.8 |
| 32 | 215 | 29 | 187 | 155 | 154 | 32 | 9.9 |
| 33 | 234 | 54 | 108 | 168 | 169 | 12 | 4.6 |
| 34 | 216 | 45 | 122 | 146 | 136 | 35 | 17.6 |
| 35 | 161 | 30 | 109 | 109 | 108 | 37 | 3.2 |
| 36 | 196 | 54 | 67 | 128 | 116 | 35 | 12.9 |
| 37 | 172 | 45 | 105 | 105 | 106 | 48 | 1.4 |
| 38 | 195 | 39 | 198 | 114 | 132 | 24 | 0.9 |
| 39 | 160 | 41 | 74 | 104 | 109 | 14 | 1.0 |
| 40 | 216 | 58 | 126 | 133 | 133 | 22 | 4.6 |
| 41 | 173 | 52 | 148 | 92 | 83 | 39 | 0.6 |
| 42 | 162 | 54 | 115 | 85 | 92 | 25 | 4.5 |
| 43 | 193 | 79 | 90 | 96 | 100 | 14 | 2.0 |
| 44 | 149 | 44 | 61 | 93 | 97 | 8 | 11.3 |
| 45 | 170 | 62 | 92 | 81 | 68 | 40 | 0.6 |
| 46 | 173 | 84 | 114 | 66 | 76 | 16 | 0.7 |
| 47 | 304 | 53 | 298 | 191 | 170 | 88 | 28.0 |
| 48 | 273 | 47 | 220 | 188 | 184 | 28 | 1.8 |
| 49 | 261 | 52 | 166 | 176 | 146 | 63 | 0.8 |
| 50 | 179 | 63 | 90 | 98 | 93 | 26 | 5.7 |
| 51 | 229 | 46 | 132 | 157 | 152 | 43 | 5.4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 52 | 224 | 39 | 152 | 152 | 149 | 46 | 2.0 |
| 53 | 208 | 40 | 201 | 128 | 140 | 28 | 2.0 |
| 54 | 267 | 37 | 122 | 189 | 175 | 55 | 16.9 |
| 55 | 189 | 51 | 78 | 122 | 103 | 38 | 0.4 |
| 56 | 225 | 60 | 110 | 143 | 134 | 27 | 0.4 |
| 57 | 208 | 79 | 51 | 119 | 121 | 8 | 2.0 |
| 58 | 172 | 77 | 44 | 86 | 78 | 19 | 1.0 |
| 59 | 152 | 57 | 43 | 87 | 87 | 13 | 1.6 |
| 60 | 254 | 78 | 66 | 163 | 147 | 31 | 6.5 |
| 61 | 194 | 51 | 140 | 115 | 104 | 59 | 1.2 |
| 62 | 172 | 59 | 117 | 89 | 78 | 44 | 0.4 |
| 63 | 165 | 59 | 117 | 104 | 83 | 33 | 3.6 |
| 64 | 193 | 53 | 61 | 114 | 105 | 54 | 14.0 |
| 65 | 151 | 57 | 66 | 81 | 59 | 36 | 5.2 |
| 66 | 200 | 33 | 115 | 144 | 137 | 58 | 1.6 |
| 67 | 241 | 32 | 470 | 115 | | | |
| 68 | 276 | 56 | 408 | 138 | | | |
| 69 | 295 | 37 | 259 | 206 | | | |
| 70 | 290 | 34 | 285 | 199 | | | |
| 71 | 310 | 43 | 595 | 148 | | | |

1 TRIG = triglyceride concentration.

2 F.E. = Friedewald Equation:
[LDL-Chol] = [Total Chol] - [HDL-Chol] - [TRIG/5].

3 U.C. = Ultracentrifuge ß-quantitation:
[LDL-Chol] = [d >1.006 Infranate-Chol] - [HDL-Chol].

4 Calculated from U.C. LDL-Chol. values.

5 Calculated by multiplying total Lp(a) measured by Terumo ELISA by 0.3: [Lp(a)-Chol] = 0.3[Total Lp(a)].

<u>8A2.1-Sepharose 4B Matrix</u>: The results are presented in Table 8 and the correlations between the immunocapture assay using MAB 8A2.1-Sepharose and the two reference methods are shown in Figure 11 (A and B). Here also lower LDL-cholesterol concentrations were obtained for some samples as compared to the reference methods. This result also demonstrates that the MAB 8A2.1 alone cannot be used

for capturing all LDL particles of heterogeneous sizes expected to be present in many subjects.

<u>4B5.6-Sepharose 4B Matrix</u>: The LDL-cholesterol concentrations determined by the two reference methods and by the immunocapture assay using MAB 4B5.6-Sepharose are presented in Table 8. The correlations between the immunocapture assay using MAB 4B5.6-Sepharose and the reference methods are shown in Figure 12 (A and B). None of the LDL-cholesterol measurements made by the immunocapture assay were substantially lower than the reference method values. These results demonstrate that the MAB 4B5.6 is capable of capturing all LDL particles of heterogeneous sizes. The correlation with the ultracentrifugation method (correlation coefficient (r) = 0.95, slope 0.93) was found to be better than with Friedewald calculation method (r = 0.95; slope 0.86). This is not surprising because it is well-known that ultracentrifugation method is superior to the Friedewald calculation method due

TABLE 8

CORRELATION BETWEEN LDL-CHOLESTEROL ASSAYS

| Samp. No. | LDL-Cholesterol (mg/dL) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | F.E.[1] | U.C.[2] | F.E. - Lp(a) | U.C. - Lp(a) | I.C.[3] 4B5.6 | I.C.[3] MB16 | I.C.[3] SPL[4] | I.C.[3] 8A2.1 |
| 1 | 99 | 97 | 98 | 96 | 96 | 97 | 97 | 92 |
| 2 | 86 | 86 | 85 | 85 | 98 | 107 | 99 | 100 |
| 3 | 61 | 61 | 59 | 59 | 50 | 63 | 64 | 53 |
| 4 | 62 | 62 | 46 | 47 | 55 | 43 | 60 | 60 |
| 5 | 57 | 64 | 42 | 49 | 50 | 50 | 54 | 55 |
| 6 | 121 | 124 | 102 | 105 | 108 | 103 | 116 | 117 |
| 7 | 121 | 122 | 102 | 103 | 122 | 110 | 127 | 116 |
| 8 | 103 | 93 | 102 | 92 | 105 | 100 | 103 | 105 |
| 9 | 59 | 73 | 52 | 66 | 62 | 61 | 54 | 70 |
| 10 | 70 | 75 | 68 | 73 | 69 | 87 | 69 | 68 |
| 11 | 80 | 92 | 75 | 87 | 89 | 99 | 91 | 97 |
| 12 | 79 | 82 | 72 | 77 | 72 | 82 | 70 | 78 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 13 | 66 | 62 | 58 | 55 | 49 | 74 | 67 | 42 |
| 14 | 113 | 103 | 107 | 97 | 107 | 120 | 130 | 101 |
| 15 | 82 | 88 | 80 | 86 | 66 | 79 | 60 | 56 |
| 16 | 79 | 81 | 69 | 71 | 72 | 81 | 36 | 30 |
| 17 | 113 | 100 | 110 | 97 | 83 | 96 | 45 | 39 |
| 18 | 65 | 68 | 64 | 67 | 63 | 85 | 32 | 26 |
| 19 | 76 | 84 | 71 | 79 | 59 | 72 | 43 | 33 |
| 20 | 39 | 35 | 38 | 34 | 34 | 50 | 27 | 32 |
| 21 | 76 | 95 | 71 | 91 | 75 | 78 | 87 | 81 |
| 22 | 113 | 121 | 104 | 112 | 107 | 115 | 120 | |
| 23 | 51 | 50 | 49 | 48 | 59 | 67 | 32 | |
| 24 | 76 | 78 | 73 | 75 | 75 | 70 | 73 | |
| 25 | 71 | 86 | 68 | 83 | 68 | 77 | 86 | |
| 26 | 70 | 60 | 68 | 52 | 62 | 59 | 74 | |
| 27 | 75 | 83 | 74 | 82 | 98 | 118 | 94 | |
| 28 | 54 | 60 | 51 | 57 | 57 | 58 | 52 | |
| 29 | 55 | 37 | 43 | 25 | 39 | 38 | 48 | |
| 30 | 105 | 106 | 101 | 102 | 91 | 103 | 107 | |
| 31 | 78 | 71 | 77 | 70 | 70 | 76 | 79 | |
| 32 | 155 | 154 | 145 | 144 | 146 | 144 | 117 | |
| 33 | 168 | 169 | 163 | 164 | 162 | 144 | 194 | |
| 34 | 146 | 136 | 129 | 119 | 130 | 126 | 170 | |
| 35 | 109 | 108 | 106 | 105 | 91 | 103 | | |
| 36 | 128 | 116 | 115 | 103 | 103 | 126 | | |
| 37 | 105 | 106 | 104 | 105 | 106 | 112 | | |
| 38 | 114 | 132 | 113 | 131 | 136 | 114 | | |
| 39 | 104 | 109 | 103 | 108 | 108 | 111 | | |
| 40 | 133 | 133 | 129 | 129 | 135 | 120 | | |
| 41 | 92 | 83 | 91 | 82 | 103 | 104 | | |
| 42 | 85 | 92 | 81 | 88 | 100 | 90 | | |
| 43 | 96 | 100 | 94 | 98 | 91 | 106 | | |
| 44 | 93 | 97 | 82 | 86 | 95 | 117 | | |
| 45 | 81 | 68 | 80 | 67 | 79 | 80 | | |
| 46 | 66 | 76 | 65 | 75 | 70 | 80 | | |
| 47 | 191 | 170 | 163 | 142 | 149 | 221 | | |
| 48 | 188 | 184 | 186 | 183 | 185 | 176 | | |
| 49 | 176 | 146 | 175 | 145 | 154 | 191 | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 50 | 98 | 93 | 92 | 88 | 91 | 94 | | |
| 51 | 157 | 152 | 152 | 147 | 140 | 145 | | |
| 52 | 152 | 149 | 150 | 147 | 145 | 162 | | |
| 53 | 128 | 140 | 126 | 138 | 125 | 133 | | |
| 54 | 189 | 175 | 172 | 158 | 157 | 174 | | |
| 55 | 122 | 103 | 122 | 103 | 106 | 110 | | |
| 56 | 143 | 134 | 143 | 134 | 134 | 129 | | |
| 57 | 119 | 121 | 117 | 119 | 100 | 99 | | |
| 58 | 86 | 78 | 85 | 77 | 73 | 70 | | |
| 59 | 87 | 87 | 85 | 85 | 77 | 88 | | |
| 60 | 163 | 147 | 156 | 140 | 128 | 148 | | |
| 61 | 115 | 104 | 113 | 103 | 105 | 118 | | |
| 62 | 89 | 78 | 89 | 78 | 93 | 98 | | |
| 63 | 104 | 83 | 100 | 80 | 80 | 79 | | |
| 64 | 114 | 105 | 100 | 91 | 89 | 115 | | |
| 65 | 81 | 59 | 76 | 54 | 65 | | | |
| 66 | 144 | 137 | 142 | 134 | 140 | | | |
| 67 | 115 | | | | 106 | | | |
| 68 | 138 | | | | 121 | | | |
| 69 | 206 | | | | 194 | | | |
| 70 | 199 | | | | 177 | | | |
| 71 | 148 | | | | 145 | | | |

[1] F.E. = Friedewald Equation:
[LDL-Chol] = [Total Chol] - [HDL-Chol] - [TRIG/5].

[2] U.C. = Ultracentrifuge ß-quantitation:
[LDL-Chol] = [d >1.006 Infranate-Chol] - [HDL-Chol].

[3] I.C. = Immunocapture assay using the named MAB.

[4] SPL = MAB SPL4A5

to the inaccuracy incurred for samples with triglyceride concentration over 300 mg/dL.

The LDL-cholesterol concentrations measured by both reference methods are actually not a true measurement of LDL-cholesterol but instead are a mixture of LDL-, IDL-, and Lp(a)-cholesterol concentrations. Although these three lipoprotein particles are considered to be potential atherogenic markers and all previous data base on LDL-

cholesterol values are based on these two reference methods, a correction for IDL and Lp(a) should be made in order to obtain a better correlation between the disease state and the true LDL-cholesterol concentrations. No reliable method of IDL quantitation is presently available except through the lengthy ultracentrifugation techniques.

A number of methods for quantitation of Lp(a) mass are known. We have used a commercial ELISA kit (TEMUMO Medial Corporation, Elkton, MD) to estimate the Lp(a) mass and the concentrations of cholesterol are then calculated by multiplying the total Lp(a) mass with 0.3, because 30% is generally assumed to be the cholesterol content of Lp(a). The Lp(a)-subtracted LDL-cholesterol concentration of the two reference methods are presented in Table 8. The LDL-cholesterol concentrations minus the Lp(a)-cholesterol contributions were correlated with the LDL-cholesterol concentrations obtained by the present immunocapture assay using MAB 4B5.6-Sepharose and are shown in Figure 13 (A and B). The correlation between immunocapture assay using MAB 4B5.6-Sepharose and ultracentrifugation method has an intercept of 0.96 a slope of 0.96. The correlation between immunocapture assay using MAB 4B5.6-Sepharose and Friedewald method has a correlation coefficient (r) of 0.95 and a slope of 0.89. Thus, the correlation of the present assay method becomes better after correcting for Lp(a)-cholesterol in the LDL-cholesterol concentrations determined by the two reference methods (see Figures 12 and 13).

The present assay method is virtually independent of the triglyceride concentration (tested up to 470 mg/dL, see Table 7) and VLDL-cholesterol (tested up to 88 mg/dL, see Table 8). The results are shown in Figures 14 (A and B) and 15 (A and B). The Y-axes in Figures 14A and 15A represent the ratio of LDL-cholesterol concentrations determined by the present method and ultracentrifugation method. The Y-axes in Figures 14B and 15B represent the ratio of LDL-cholesterol concentrations determined by the present method and ultracentrifugation method corrected for the Lp(a)-cholesterol contribution. The

TABLE 9

LDL-CHOLESTEROL IN LIPID LOWERING DRUG TREATED PATIENTS

| Sample No. | Total Chol. mg/dL | HDL-Chol. mg/dL | TRIG[1] mg/dL | F.E.[2] LDL-Chol. mg/dL | U.C.[3] LDL-Chol. mg/dL | I.C.[4] LDL-Chol. mg/dL |
|---|---|---|---|---|---|---|
| 72 | 205 | 37 | 275 | 113 | 112 | 125 |
| 73 | 210 | 26 | 425 | N/A | 78 | 93 |
| 74 | 218 | 47 | 170 | 137 | 121 | 132 |
| 75 | 257 | 45 | 222 | 168 | 168 | 163 |
| 76 | 249 | 35 | 136 | 187 | 169 | 174 |
| 77 | 151 | 38 | 230 | 67 | 56 | 76 |
| 78 | 299 | 43 | 175 | 221 | 224 | 209 |
| 79 | 323 | 65 | 430 | N/A | 204 | 161 |
| 80 | 180 | 30 | 242 | 102 | 100 | 103 |
| 81 | 275 | 40 | 177 | 200 | 191 | 174 |
| 82 | 289 | 41 | 320 | 184 | 173 | 179 |
| 83 | 332 | 61 | 140 | 209 | 219 | 184 |
| 84 | 208 | 45 | 129 | 137 | 131 | 128 |
| 85 | 225 | 49 | 193 | 137 | 132 | 123 |
| 86 | 201 | 44 | 114 | 134 | 123 | 115 |
| 87 | 220 | 69 | 96 | 119 | 118 | 112 |
| 88 | 436 | 46 | 336 | 325 | 166 | 203 |
| 89 | 341 | 43 | 701 | N/A | 147 | 186 |
| 90 | 798 | 53 | 679 | N/A | 329 | 451 |
| 91 | 361 | 27 | 903 | N/A | 195 | 164 |

[1] TRIG = triglyceride concentration.

[2] F.E. = Friedewald Equation:
[LDL-Chol] = [Total Chol] - [HDL-Chol] - [TRIG/5].

[3] U.C. = Ultracentrifuge ß-quantitation:
[LDL-Chol] = [d >1.006 Infranate-Chol] - [HDL-Chol].

[4] I.C.= Immunocapture assay using MAB 4B5.6.

N/A = F.E. LDL-cholesterol cannot be used for individuals with high TRIG levels (greater than about 400 mg/dL).

X-axes in Figures 14 and 15 represent triglyceride and VLDL concentration, respectively.

The present assay method for LDL-cholesterol was evaluated with patient samples who are on lipid lowering drugs. The results are presented in Table 9.

MB16-Sepharose 4B Matrix: The LDL-cholesterol concentrations measured by the immunocapture assay using MAB MB16-Sepharose and by the two reference methods are presented in Table 8. Figure 16 (A and B) shows the correlations between the immunocapture assay using MAB MB16-Sepharose and ultracentrifugation and Friedewald methods. The correlation between immunocapture assay using MAB MB16-Sepharose and ultracentrifugation method has a correlation coefficient (r) of 0.91 a slope of 0.96. The correlation between immunocapture assay using MAB MB16-Sepharose and Friedewald method has a correlation coefficient (r) of 0.93 and a slope of 0.90. Figure 17 (A and B) shows the correlations after the Lp(a)-cholesterol contribution has been subtracted from the two reference methods. In this study also, the correlations become better after Lp(a)-cholesterol correction similar to that observed for the MAB 4B5.6-Sepharose immunocapture assay.

The immunocapture assay using MAB MB16-Sepharose showed no dependency on the triglyceride concentration (tested up to 333 mg/dL; see Table 7). The results are shown in Figure 18 (A and B). However, the immunocapture assay using MAB MB16-Sepharose showed some dependency on VLDL-cholesterol (see Table 7). The results are shown in Figure 19 (A and B). This dependency is not unexpected because MAB MB16 showed about 19% cross-reactivity with VLDL compared to only 7% for MAB 4B5.6 in the competitive RIA (see Table 3).

The Y-axes in Figures 18A and 19A represent the ratio of LDL-cholesterol concentrations determined by the present method and ultracentrifugation method. The Y-axes in Figures 18B and 19B represent the ratio of LDL-cholesterol concentrations determined by the present method and ultracentrifugation method corrected for the Lp(a)-

cholesterol contribution. The X-axes in Figures 18 and 19 represent triglyceride and VLDL concentration, respectively.

## 6. CHOLESTEROL BINDING AGENTS

### a. LDL-Cholesterol Standards

Plasma samples of known LDL-cholesterol concentrations as determined by reference methods were used to generate standard curves. Standards with LDL-cholesterol concentrations of 74, 101, 135 and 207 mg/mL were prepared by diluting the plasma samples with 1% alkali-treated casein in 20 mM phosphate buffered saline (PBS) at pH 7.4.

### b. Preparation of Digitonin-Peroxidase Conjugates

Digitonin (2.5 mg/mL in water) (water soluble containing 50% digitonin and sodium deoxycholate commercially available from Sigma Chemical Company, St. Louis, MO) was oxidized with sodium meta-periodate (a solution of 1.68% w/v periodate in water was added to the digitonin solution to a final concentration of 0.02 M periodate) (Tschesche and Wulff (1963) Tetrahedron 19:621-634). The mixture was stirred at 4°C for one hour and then dialyzed against 20 mM phosphate buffered saline (PBS), pH 8.0, at 4°C overnight. The oxidized digitionin was then mixed with ethylenediamine (a solution of 0.25 M ethylenediamine in 20 mM PBS, at pH 8.0, was added to the oxidized digitonin solution to a final concentration of 0.05 M ethylenediamine) and incubated at 4°C. The mixture was then reduced by two additions of 100 µL of 4 mg/mL sodium borohydride in 0.1 N sodium hydroxide (i.e. 100 µL of the sodium borohydride solution per 30 mg of digitonin), after 30 minutes and after 60 minutes. After incubating at 4°C for two hours, the mixture was dialyzed against 0.01 M carbonate buffer, pH 9.5, at 4°C overnight.

Five milligrams (5 mg) of horseradish peroxidase (HRPO) (155 Ku/mg, commercially available from Amano International) were dissolved in water to a final

concentration of 4 mg/mL HRPO. The HRPO was oxidized by adding 50µL of a freshly prepared solution of 0.2 M sodium meta-periodate per milligram of HRPO to the HRPO solution and incubating the mixture in the dark at room temperature for 20 minutes. The mixture was then dialyzed against 2 liters of 1 mM acetate buffer, pH 4.5, at 4°C for 4 hours.

The ethylenediamine derivatized digitonin solution and the oxidized HRPO solution were mixed in digitonin:HRPO weight ratios of 1:5 and 1:10. To each reaction was added 0.2 M carbonate buffer, pH 9.5 (50 µL buffer/mg digitonin), and the pH was adjusted to 9.5 as necessary. The reactions were stirred in the dark at room temperature for two hours and 100 µL of sodium borohydride solution (4 mg/mL in water) was added to each reaction. After incubating for two hours at 4°C, the reactions were dialyzed against 20 mM PBS, pH 7.4, at 4 °C overnight. To each mixture was added 5% (w/v) of sodium deoxycholate in water (one-tenth the volume of the dialyzed solutions) and fatty-acid free bovine serum albumin (to a final concentration of 5 mg/mL). The solutions were then sterile filtered through a 0.22 micro filter (Coaster Labs) and stored at -20°C.

c. Preparation of Tomatine-Peroxidase Conjugates

Tomatine-HRPO conjugates in weight ratios of 1:5 and 1:10 were prepared from tomatine (Sigma Chemical Company, St. Louis, MO) (Reichstein (1962) Agnew Chem. 74:887-918) dissolved in 1% (w/v) aqueous sodium deoxycholate using the same procedure described above for digitonin-HRPO conjugates (paragraph 6.b.).

d. Preparation of Amphotericin-Peroxidase Conjugates

Amphotericin-HRPO conjugates in weight ratios of 1:5 and 1:10 were prepared from 1 mg amphotericin B (80% pure, Sigma Chemical Company, St. Louis, MO) suspended in 1 mL of 0.01 M bicarbonate buffer using the same procedure described above for digitonin-HRPO conjugates (paragraph 6.b.). The conjugates were sterile filtered using 0.45 micro filters.

e. Preparation of Anti-Cholesterol Antibodies

3-Hydroxycholan-5-en-24-oic acid (commercially available from Steraloids Inc., Wilton, NH) was reacted with N-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide to form an active ester under typical reaction conditions. The active ester was reacted with 6-aminohexanoic acid to form 6-(3-hydroxycholan-5-en-24-carbonylimino)hexanoic acid. This carboxylic acid was reacted with N-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide to form a second active ester which was reacted with bovine serum albumin to form the immunogen. The immunogen was purified on a Sephadex G-25 column using standard purification techniques.

3-Hydroxycholan-5-en-24-oic acid may be coupled directly to natural or synthetic proteins to form immunogens useful in the preparation of both polyclonal and monoclonal anti-cholesterol antibodies. Other amino acid linking groups like aminohexanoic acid, such as aminoacetic acid, aminopropanoic acid, aminoheptanoic acid and the like, may be used to prepare useful immunogens. Also, diamino linking groups, such as ethylenediamine and the like, can be used to prepare useful immunogens. Linker arms of the general formula $X\text{-}(CH_2)_n\text{-}Y$, where X is a primary or secondary amine or a carboxylic acid group, Y is a primary or secondary amine and $n = 1\text{-}10$, are useful for the preparation of anti-cholesterol producing immunogens. The steroid with such a linking group can be coupled to natural or synthetic proteins to form immunogens useful in the preparation of both polyclonal and monoclonal anti-cholesterol antibodies.

The preparation of polyclonal and monoclonal antibodies using immunogens is well known in the art (Tijssen, "Laboratory Techniques In Biochemistry And Molecular Biology: Practice and Theory of Enzyme Immunoassays", Vol. 15, Elsevier, New York (1985)).

Polyclonal antibodies were preferably raised in rabbits, but other animals, such as sheep, pigs, mice, rats, goats, donkeys and the like, can also produce suitable antibodies.

Antibody binding was tested by enzyme-linked immunosorbent assay. Cholesterol antigens, such as HDL, LDL, VLDL and IDL particles in 0.15 M saline, cholesterol in 95% ethanol (0.25 μg/well) and cholesterol esters in hexane (0.25 μg/well), were absorbed in polystyrene microtiter plate wells. The wells were then blocked with 10% (v/v) fetal calf serum or 1% (w/v) casein solutions. The antibody to be tested was incubated in the well and the well was washed with 0.05% (w/v) Tween 20 in 20 mM PBS, pH 7.0. Enzyme labelled anti-antibody antibody, such as goat anti-rabbit IgG conjugated to HRPO, was used to detect the presence of antibody bound to the cholesterol antigen absorbed in the well. Rabbit antibody raised against the 6-(3-hydroxycholan-5-en-24-carbonylimino)hexanoic acid hapten coupled to BSA bound to cholesterol and cholesterol esters, but showed preferential binding to lipoprotein cholesterol particles in the order: HDL ≈ VLDL > LDL > IDL.

f. Preparation of Antibody-Peroxidase Conjugates

Anti-cholesterol Antibody-HRPO conjugates were prepared as follows. Anti-cholesterol rabbit polyclonal antibody was purified by double precipitation with 33% saturated ammonium sulfate. The antibody (2.7 mg of IgG) was coupled to HRPO (1.44 mg) using the procedure described above for digitonin-HRPO conjugates (paragraph 6.b.).

g. Preparation of Digitonin-Phosphatase Conjugates

Calf intestine alkaline phosphatase (AP) (10 mg/mL) was mixed with sodium meta-periodate (4.28 mg/mL) in 0.2 M sodium acetate, pH 4.5, and stirred at room temperature in the dark for three hours. The mixture was desalted on a Pharmacia PD-10 column which was pre-equilibrated with a solution of 10 mM sodium acetate, 0.1 M sodium chloride, 1 mM magnesium chloride and 0.1 mM zinc chloride at pH 4.5. The oxidized alkaline phosphatase was then mixed with the ethylenediamine derivatized digitonin ( 0.6 mL of a 1.67 mg/mL solution in 20 mM PBS, pH 7.4) prepared above

(paragraph 6.b.) in 20 mM PBS, pH 7.4 in a digitonin:phosphatase weight ratio of 1:5. To the mixture was added 90 μL of 1 M bicarbonate buffer, pH 9.5 and the mixture was incubated at room temperature for 16 hours in the dark. Thirty microliters of sodium borohydride (5 mg/mL in 0.1 M bicarbonate buffer, pH 9.5) was added to the reaction and the reaction was incubated at 4°C for 4 hours. The reaction was then dialyzed against 2 liters of a solution of 0.05 M TRIS, 0.1 M sodium chloride, 1 mM magnesium chloride, 0.1 mM zinc chloride and 0.1% (w/v) sodium azide, pH 8.0, at 4°C overnight. A solution of 5% sodium deoxycholate in water (one-tenth the volume of the dialyzed material) and fatty-acid free BSA were added to make the BSA final concentration 5 mg/mL. The digitonin-AP conjugate was sterile filtered through a 0.22 micro filter and stored at -20°C.

h. Preparation of Other Conjugates

Tomatine-alkaline phosphatase conjugates were prepared in tomatine:phosphatase weight ratios of 1:5 and 1:10 according to the digitonin-AP conjugate procedure (paragraph 6.g.). Amphotericin-alkaline phosphatase conjugates were prepared in amphotericin:phosphatase weight ratios of 1:5 and 1:10 according to the digitonin-AP conjugate procedure (paragraph 6.g.). Antibody-alkaline phosphatase conjugates were prepared from rabbit anti-cholesterol IgG antibody (2.4 mg IgG) and AP (7.2 mg) according to the digitonin-AP conjugate procedure (paragraph 6.g.).

i. Peroxidase Conjugate Binding to LDL

Maxisorb Nunc Immuno plates were coated with 100 μL of pure LDL (5 μg/mL cholesterol concentration) in 20 mM PBS, pH 7.4, at 37°C for 30 minutes. After blocking the non-specific binding sites with 200 μL of 5% BSA in 20 mM PBS, pH 7.4, at 37°C for one hour, the plates were washed five times with 0.05% Tween 20 in 20 mM PBS, pH 7.4 (PBS-Tween). Then each conjugate was titrated from 5 μg/mL in 2% BSA in 20 mM PBS, pH 7.4 (100 μL in each well). The plates

were incubated at 37°C for one hour and washed eight times with PBS-Tween. o-Phenylenediamine (OPD) (100 µL of a standard solution prepared from one OPD tablet/5 mL citrate buffer, pH 6; both commercially available from Abbott Laboratories, IL) was added to the wells. After incubation for 5 minutes, the color reaction was stopped with 100 µL of 1 N sulfuric acid. The plates were read at 490 nm on a microplate reader (Bio-Tek). At a conjugate concentration of 1.25 µg/mL, the following absorbances were observed: digitonin-HRPO (1:5) = 2.2; digitonin-HRPO (1:10) = 1.23; tomatine-HRPO (1:5) = 1.2; tomatine-HRPO (1:10) = 1.35; amphotericin B-HRPO (1:5) = 0.2; and amphotericin B-HRPO (1:10) = 0.18. At a conjugate concentration of 10 µg/mL, amphotericin B-HRPO (1:5 or 1:10) gave an absorbance of 0.8. Rabbit antibody-HRPO conjugate gave an absorbance of 0.6 at a conjugate concentration of 1.25 µg/mL and a 1.70 absorbance at a conjugate concentration of 10 µg/mL.

j. Phosphatase Conjugate Binding to LDL

Maxisorb Nunc Immuno plates were coated with 100 µL of pure LDL (5 µg/mL cholesterol concentration) in 20 mM PBS, pH 7.4, at 37°C for 30 minutes. After blocking the non-specific binding sites with 200 µL of 5% BSA in 20 mM PBS, pH 7.4, at 37°C for one hour, the plates were washed five times with 50 mM TRIS, 150 mM sodium chloride, 0.1% sodium azide and 0.05% Tween 20, pH 7.4 (TRIS-Tween). Then each conjugate (100 µL in each well) was titrated from 20 µg/mL in 1% alkali-treated casein in 50 mM TRIS, 100 mM sodium chloride, 1 mM magnesium chloride, 0.1 mM zinc chloride, 0.1% sodium azide, pH 8.0 (dilution buffer). The plates were incubated at 37°C for one hour and washed eight times with TRIS-Tween. p-Nitrophenolphosphate (100 µL of 2 mg/mL in dilution buffer) was added to each well. After incubation at room temperature for 16 minutes, the color reaction was stopped with 100 µL of 1 N sodium hydroxide. The plates were then read at 405 nm on a microplate reader. At a conjugate concentration of 10 µg/mL, the following absorbances were

observed: digitonin-AP (1:5) = 1.52; digitonin-AP (1:10) = 1.4; tomatine-AP (1:5) = 1.55; tomatine-AP (1:10) = 1.65; and amphotericin B-AP (1:5 OR 1:10) = 0.15. Rabbit antibody-AP conjugate gave an absorbance of 0.7 at a conjugate concentration of 10 μg/mL and a 0.97 absorbance at a conjugate concentration of 20 μg/mL.

k. Anti-LDL Coated Plates

The LDL specific monoclonal antibody 4B5.6 was diluted in 20 mM PBS, pH 7.4, to a final concentration of 5 μg/mL. One-hundred microliters of the solution was added to each well of Maxisorb Nunc Immuno plates and incubated at room temperature with gentle shaking for two hours. The plates were washed five times with PBS-Tween and then blocked with 200 μL of 5% BSA in 20 mM PBS by incubation at 37°C for one hour. The plates were stored at 4°C with plastic sealers. Before use, the plates were washed five times with PBS-Tween for HRPO conjugates and TRIS-Tween for AP conjugates.

l. LDL-Cholesterol Standard Curves

LDL-cholesterol standards (100 μL/well in duplicate) were incubated in the 4B5.6 plates (paragraph 6.k.) at 37°C for one hour. After washing the plates five times with PBS-Tween, 100 μL of digitonin-HRPO conjugate at 0.4 μg/mL in 1% casein in PBS or at 1.25 μg/mL in 1% casein in PBS was added to each well and incubated at 37°C for one hour. The plates were washed with PBS-Tween eight times. o-Phenylenediamine (OPD) (100 μL of a standard solution prepared from one OPD tablet/5 mL citrate buffer, pH 6; both commercially available from Abbott Laboratories, IL) was added to the wells. After incubation for 5 minutes, the color reaction was stopped with 100 μL of 1 N sulfuric acid. The plates were read at 490 nm on a microplate reader (Bio-Tek). Standard curves (absorbance vs. LDL-cholesterol concentration) were then prepared from the results. Standard

curves were also prepared with the tomatine-HRPO conjugates.

TABLE 10

CORRELATION BETWEEN LDL-CHOLESTEROL ASSAYS

| Samp. No. | Total Chol. mg/dL | TRIG[1] mg/dL | LDL-CHOLESTEROL (mg/dL)[4] | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | F.E.[2] | U.C.[3] | DIG-HRPO | DIG-AP | TOM-HRPO | TOM-AP |
| 201 | 255 | 77 | 192 | 183 | 183 | 191 | 189 | 191 |
| 202 | 143 | 49 | 83 | 80 | 94 | 93 | 92 | 73 |
| 203 | 141 | 40 | 74 | 70 | 73 | 74 | 75 | 74 |
| 204 | 198 | 53 | 123 | 116 | 108 | 110 | 108 | 109 |
| 205 | 160 | 52 | 89 | 85 | 113 | 94 | 92 | 85 |
| 206 | 201 | 82 | 144 | 142 | 134 | 130 | 136 | 136 |
| 207 | 167 | 45 | 102 | 98 | 102 | 97 | 101 | 99 |
| 208 | 197 | 127 | 135 | 130 | 138 | 137 | 133 | 132 |
| 209 | 247 | 119 | 167 | 164 | 162 | 165 | 162 | 158 |
| 210 | 186 | 83 | 116 | 112 | 103 | 120 | 122 | 118 |
| 211 | 230 | 93 | 160 | 157 | 167 | 157 | 157 | 164 |
| 212 | 237 | 243 | 154 | 160 | 190 | 175 | 190 | 199 |
| 213 | 280 | 164 | 202 | 197 | 206 | 207 | 207 | 211 |
| 214 | 168 | 79 | 114 | 114 | 121 | 119 | 122 | 122 |
| 215 | 215 | 187 | 155 | 154 | 143 | 139 | 146 | 147 |
| 216 | 216 | 122 | 146 | 136 | 138 | 152 | 152 | 155 |
| 217 | 162 | 148 | 85 | 92 | 130 | 129 | 144 | 133 |
| 218 | 149 | 61 | 93 | 97 | 111 | 129 | 142 | 124 |
| 219 | 170 | 92 | 81 | 68 | 132 | 129 | 130 | 123 |
| 220 | 173 | 114 | 70 | 76 | 113 | 95 | 89 | 99 |
| 221 | 273 | 220 | 188 | 184 | 135 | 145 | 137 | 143 |
| 222 | 179 | 90 | 98 | 93 | 89 | 108 | 93 | 90 |
| 223 | 229 | 132 | 157 | 152 | 147 | 156 | 139 | 152 |
| 224 | 224 | 152 | 152 | 149 | 135 | 126 | 128 | 122 |
| 225 | 267 | 122 | 189 | 175 | 158 | 167 | 156 | 145 |
| 226 | 189 | 78 | 122 | 103 | 117 | 131 | 121 | 132 |
| 227 | 225 | 110 | 143 | 134 | 141 | 166 | 141 | 153 |
| 228 | 208 | 51 | 119 | 121 | 120 | 115 | 131 | 130 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 229 | 152 | 43 | 87 | 87 | 109 | 118 | 118 | 117 |
| 230 | 354 | 66 | 163 | 143 | 162 | 179 | 134 | 167 |
| 231 | 194 | 140 | 115 | 104 | 96 | 110 | 123 | 117 |
| 232 | 172 | 117 | 89 | 78 | 98 | 93 | 109 | 94 |
| 233 | 193 | 61 | 114 | 105 | 133 | 121 | 148 | 129 |
| 234 | 172 | 51 | 86 | 78 | 89 | 108 | 111 | 80 |
| 235 | 218 | 170 | 137 | 121 | 133 | 128 | 161 | 129 |
| 236 | 257 | 222 | 168 | 168 | 158 | 156 | 205 | 163 |
| 237 | 151 | 230 | 67 | 56 | 62 | 65 | 82 | 63 |
| 238 | 299 | 175 | 221 | 224 | 165 | 194 | 205 | 138 |
| 239 | 323 | 430 | | 204 | 133 | 164 | 163 | 122 |
| 240 | 180 | 242 | 102 | 100 | 96 | 120 | 107 | 92 |
| 241 | 275 | 177 | 200 | 191 | 190 | 194 | 200 | 185 |
| 242 | 289 | 320 | 184 | 173 | 181 | 167 | 183 | 160 |
| 243 | | | | | 164 | 132 | 185 | 161 |
| 244 | 332 | 140 | 209 | 219 | 189 | 213 | 205 | 203 |
| 245 | 208 | 129 | 137 | 131 | 110 | 127 | 126 | 127 |
| 246 | 225 | 193 | 137 | 132 | 110 | 126 | 140 | 130 |
| 247 | 210 | 114 | 134 | 123 | 132 | 153 | 143 | 138 |
| 248 | 220 | 96 | 119 | 118 | 91 | 107 | 83 | 86 |
| 249 | | | | | 128 | 128 | 185 | 127 |
| 250 | 117 | 51 | 79 | 82 | 83 | 94 | 54 | 82 |
| 251 | 172 | 70 | 113 | 121 | 132 | 131 | 137 | 135 |
| 252 | 436 | 336 | | 166 | 262 | 212 | 265 | 235 |
| 253 | 345 | 328 | | 115 | 122 | 125 | 106 | 143 |
| 254 | 337 | 468 | | 99 | 93 | 101 | 69 | 113 |
| 255 | 341 | 701 | | 147 | 197 | 164 | 235 | 170 |

1 TRIG = triglyceride concentration.

2 F.E. = Friedewald Equation:
[LDL-Chol] = [Total Chol] - [HDL-Chol] - [TRIG/5].

3 U.C. = Ultracentrifuge ß-quantitation:
[LDL-Chol] = [d >1.006 Infranate-Chol] - [HDL-Chol].

4 DIG-HRPO = digitonin-HRPO conjugate based assay; DIG-AP = digitonin-AP conjugate based assay; TOM-HRPO = tomatine-HRPO conjugate based assay; and TOM-AP = tomatine-AP conjugate based assay.

Standard curves were also prepared with the digitonin-AP and tomatine-AP conjugates using the same procedure except that the TRIS-Tween wash, the dilution buffer and p-nitrophenolphosphate substrate (100 µL of 2 mg/mL in dilution buffer) were used as in paragraph 6.j. A 16 minute substrate incubation was used and the reaction was stopped with 100 µL of 1 N sodium hydroxide. The absorbances were read at 405 nm.

m. Evaluation of LDL-Cholesterol Sandwich Assay

Plasma samples in ethylenediaminetetraacetic acid (EDTA) were collected from normal individuals and patients. The samples were frozen at -20°C until used. Thawed samples were not used after two days storage at 4°C. The samples were diluted 600-fold in 1% casein in PBS and assayed for LDL-cholesterol using the procedures of paragraph 6.l. wherein the diluted samples were used in place of the standards. Along with the samples, standards were also assayed in duplicate as before. For each microtiter plate, a standard curve was generated and the values of the samples were determined using a point-to-point fitted computer program. The LDL-cholesterol measurements of the samples are shown in Table 10.

These results demonstrate the efficacy of the present invention. The present invention is suitable for quantitating cholesterol and cholesterol esters associated with lipoprotein particles by the use of lipoprotein specific antibodies. The following more detailed examples are intended to further illustrate the present invention.

## EXAMPLE 1

## PURIFICATION OF MONOCLONAL ANTIBODIES

Purification of the monoclonal antibodies was performed on Protein A-Sepharose 4B columns (Pharmacia LKB). The IgG fractions were first precipitated with 50% ammonium sulfate and the precipitate was re-dissolved and dialyzed against 20

mM phosphate buffered saline (PBS), pH 7 4°C. The dialyzed sample was diluted with an equal volume of 1.5M glycine in 3M sodium chloride at pH 8.9 (binding buffer) and loaded onto a Protein A column. A column size of 1.25 mL/mL of ascites was used in the purification process. The flow rate during sample loading was maintained at about 0.5 mL/minute. The column was washed with binding buffer until the absorbance at 280 nm is ≤ 0.02. The bound IgG antibody was then eluted sequentially with 100 mM citrate buffer, pH 6 (for $IgG_1$), pH 5 (for IgG2a) and pH 4 (for IgG2b). Ten milliliters of each elution buffer was used for each milliliter of protein A in the column. The column was regenerated by washing with 100 mM citrate buffer, (pH 3) until the absorbance at 280 nM is ≤0.02 and then re-equilibrated with the binding buffer. Each column was used at least five times without any loss of binding affinity.

## EXAMPLE 2

## PREPARATION OF LIPOPROTEIN FRACTIONS

Blood samples from normal fasting subjects were collected into ethylenediaminetetraacetic acid (EDTA) and the red blood cells were removed by centrifugation. The plasma samples were then analyzed for Lp(a) using TERUMO ELISA kit. Plasma samples containing less than 1 mg/dL Lp(a)-cholesterol were selected for the purification of VLDL, IDL, LDL and HDL. Lipoprotein subtractions were prepared in a Beckman Ultracentrifuge with a SW 40 Ti rotor by successive ultracentrifugation at 4°C (Havel et al. (1955) *J. Clin. Invest.* 34:1345-1355). VLDL was collected at a density of about d 1.0006 g/mL; IDL was collected at a density range of about d 1.006-1.019 g/mL; LDL was collected at a density range of about d 1.019-1.050 g/mL; and HDL was collected at a density range of about d 1.080-1.225 g/mL. All fractions were isolated by a tube-slicing technique. The lipoprotein fractions were dialyzed exhaustively against 0.15 M sodium chloride containing 0.1% EDTA and 0.1% sodium azide, pH 7.4 at 4°C. IDL, LDL and HDL fractions were sterile filtered through 0.2 micron

and VLDL through 0.45 micron membrane filters (Nalgene) and stored at 4°C. Lipoprotein (a) was isolated from plasma samples with Lp(a)-cholesterol concentrations more than 15 mg/dL on a lysine-Sepharose column (Fless and Scanu, *Arteriosclerosis* (1987) 7:505A). The purity of each lipoprotein fraction was evaluated by electrophoresis under non-denaturing polyaccrylamide gradient gel electrophoresis (Lefevre et al. (1987) *J. Lipid Res.* 28:1495-1509). Gradient slab gels, 2-16% and 4-30% and electrophoresis apparatus GE-24 (Pharmacia LKB) were used in the analysis. The lipoprotein fractions containing no cross-contamination were used in the studies.

EXAMPLE 3

PREPARATION OF PEROXIDASE CONJUGATE OF ANTI-LDL MONOCLONAL ANTIBODY

Horseradish peroxidase (1 mg = 155 Ku, Amano International) was dissolved in water (250 µL) and oxidized with freshly prepared 0.2 M sodium m-periodate (50 µL) at room temperature in the dark for 20 minutes. The oxidized peroxidase was then dialyzed against 2 liters of 1 mM acetate buffer (pH 4.5) at 4°C for four hours. Monoclonal antibody B06 (1.9 mg/mL), which was dialyzed against 0.01 M carbonate buffer (pH 9.5) at 4°C, was treated with 20 µL of 0.2 M carbonate buffer (pH 9.5). The antibody and the dialyzed peroxidase were then mixed at room temperature in the dark for two hours. To this mixture 24 µL of freshly prepared sodium borohydride (Aldrich, 4 mg/mL in water) was added and then incubated at 4°C in the dark for two hours. The peroxidase-antibody conjugate was then dialyzed against two liters of 20 mM phosphate buffered saline (pH 7.4) at 4°C and stored at -20°C in small aliquots.

The binding curves of B06-peroxidase conjugate to lipoproteins are shown in Figure 3. A Maxisorb Nunc Immuno plate was coated with 100 µL of different lipoproteins by incubation at 37°C for 1/2 hour. After blocking the non-specific sites with 200 µL of 10% FBS in PBS at 37°C for one

hour, and washing five times with PBS-Tween 20, 100 μL of B06-peroxidase conjugate (0.6 μg/mL diluted in 3% FBS in PBS) was added to each well. The plate was incubated at 37°C for 1/2 hour, washed eight times with PBS-Tween 20. One hundred microliters of OPD substrate solution was added to each well. After incubation at room temperature for five minutes, the color reaction was stopped with 100 μL of 1N $H_2SO_4$. The plate was then read at 490 nm on a microplate reader.

EXAMPLE 4

COVALENT ATTACHMENT OF MONOCLONAL ANTIBODY TO CNBr-ACTIVATED SEPHAROSE 4B

One gram of CNBr-activated Sepharose 4B (Pharmacia LKB) was suspended in about 15 mL of 1 mM HCl. The gel was then transferred to a coarse-porosity sintered-glass funnel and washed with about 200 mL of 1 mM HCl. The gel was then washed with 25 mL of 0.1 M carbonate buffer in 0.5 M sodium chloride, pH 8.3 (coupling buffer). A gentle vacuum was applied to remove the buffer. The moist gel cake was then transferred to a glass tube with a screw-capped stopper. Monoclonal antibody (10 mg, concentration 0.5 to 1 mg/mL), which was dialyzed against the coupling buffer at 4°C, was then added to the gel. The mixture was then mixed gently end-over-end using an infiltration wheel at 4°C for 20 hours. The supernatant was checked by measuring the absorbance at 280 nm for the unbound antibody. For all the monoclonal antibodies used here, more than 95% were bound to the gel. The gel was then transferred to a coarse-porosity sintered-glass funnel, washed with 50 mL of the coupling buffer and 25 mL of 0.1 M TRIS-HCl buffer, pH 8.0 (blocking buffer). The gel was then transferred to a glass tube, and mixed with 10 mL of the blocking buffer at room temperature for two hours. The antibody-immobilized gel was then washed with three cycles of alternating pH. Each cycle consisted of a wash with acetate buffer (0.1 M, pH 4) containing sodium chloride (0.5 M) followed by a wash with TRIS buffer (0.1 M, pH 8) containing

sodium chloride (0.5 M) The final wash was done with 100 mL of TRIS-HCl buffer (0.05 M pH 7.4) containing sodium chloride (0.15 M) and sodium azide (0.01%) (storage buffer). The gel was stored as a 25% suspension (14 mL) in the storage buffer at 4°C. Assuming 100% of the monoclonal antibody bound to the gel, 200 μL of gel suspension contains 143 μg of the monoclonal antibody.

EXAMPLE 5

COVALENT ATTACHMENT OF MONOCLONAL ANTIBODY TO HYDRAZIDE GEL

Six milliliters of hydrazide gel (Pierce Chemicals, Carbolink hydrazide, 50% suspension) were washed with 50 mL of 0.1 M phosphate buffer (pH 7.0) in a coarse-porosity sintered-glass funnel. After a gentle vacuum to remove the buffer, the moist gel was transferred to a glass tube with a screw-capped stopper. Five milligrams of monoclonal antibody (concentration, 2 mg/mL), which was dialyzed against 0.1 M phosphate buffer (pH 7.0) at 4°C, was oxidized with 10.5 mg sodium m-periodate at room temperature for 1/2 hour. The oxidized antibody (volume 2.5 mL) was then loaded on a Sephadex G-25 M PD-10 column (Pharmacia) which was pre-equilibrated with 0.1 M phosphate buffer (pH 7.0). The oxidized antibody was eluted with 3 mL of 0.1 M phosphate buffer (pH 7.0) and mixed with the hydrazide gel end-over-end using an infiltration wheel at room temperature for seven hours. The supernatant was checked for the unbound antibody. The amount of antibody bound to the gel ranged between 80-85%. The antibody-immobilized gel was filtered through a coarse-porosity sintered-glass funnel, washed with about 100 mL of 1 M sodium chloride and finally, with 0.05 M TRIS-HCl (pH 7.4) containing 0.15 M sodium chloride and 0.01% sodium azide (storage buffer). The immobilized gel was stored as a 25% suspension in the storage buffer (12 mL) at 4°C. Assuming 80% of antibody bound to gel 400 μL suspension contains about 133 μg of antibody.

EXAMPLE 6

COVALENT ATTACHMENT OF MONOCLONAL ANTIBODY TO SULFO GEL

Six milliliters of sulfolink gel (Pierce Chemicals, 50% suspension) were washed in a coarse-porosity sintered-glass funnel with 50 mL of 0.05 M TRIS buffer (pH 8.5) containing 0.005 M EDTA (reaction buffer). After a gentle vacuum to remove the buffer, the moist cake was transferred to a glass tube with a screw-capped stopper. Five milligrams of monoclonal antibody (2 mg/mL), which was dialyzed against 0.1 M phosphate buffer (pH 6) containing 0.005 M EDTA at 4°C, was reduced with 12 mg of 2-mercaptoethylamine HCl (Pierce Chemicals) at 37°C for 1 1/2 hours. The reaction mixture was cooled to room temperature and loaded onto a Sephadex G-25M PD-10 column (Pharmacia) which was pre-equilibrated with the reaction buffer. The reduced antibody was then eluted with 3 mL of the reaction buffer and mixed with the sulfolink gel end-over-end using an infiltration wheel at room temperature for 15 minutes. The reaction mixture was allowed to stand at room temperature for an additional hour. The supernatant was checked for the unbound antibody. The amount of antibody bound to the gel ranged between 75-80%. The gel was filtered through a coarse-porosity sintered-glass funnel, washed with 50 mL of the reaction buffer and again transferred to a glass tube. Three milliliters of 0.05 M cysteine in reaction buffer was added to the gel and mixed end-over-end at room temperature for one hour. The gel was transferred to a coarse-porosity sintered-glass funnel and washed with 50 mL of 1 M sodium chloride and finally, with 50 mL of PBS (pH 7) containing 0.02% sodium azide, (storage buffer). The immobilized gel was stored as a 25% suspension in the storage buffer at 4°C (12 mL). Assuming 75% of antibody bound to the gel, 400 μL of the gel suspension contains 125 μg of antibody.

EXAMPLE 7

PREPARATION OF THE CHOLESTEROL ASSAY REAGENTS

a. Dry Reagents: Methods and formulation are described here to produce sensitive, rapid and stable assay reagents for the quantitation of cholesterol in a fluid phase. Two separate reagents were prepared and were mixed together at the time of the assay. The first reagent formula was comprised of 1.62 g of 3,5-dichloro-2-hydroxybenzenesulfonic acid sodium salt (Aldrich, Milwaukee, Wisconsin) (DCHBS) and 0.428 g of horseradish peroxidase (Amano International, specific activity 82.3 EZ/mg) (HRPO) dissolved in 20.4 mL 0.05 M of 3-(N-Morpholino)-2-hydroxypropanesulfonic acid sodium salt (Sigma) (MOPSO) at pH 7. The solution was then added to 15.8 g of custom made LC06 ww 5.3 frits (Porex Industries; each frit weighed 7.9 mg, volume 8.4 μL) in a brown bottle and mixed end-over-end for 15 minutes. The entire container was then lyophilized to complete dryness. The final moisture content of each frit must be ≤1% as determined by Karl Fisher autotitrator. The first reagent frits were stored dry in a brown bottle at room temperature with some added molecular sieve pouches as a desiccant (Multiform Desiccants). Each frit weighed 8.86 mg on average and contained 0.672 g DCHBS, 0.1786 g HRPO, and 0.1051 g MOPSO. The peroxidase activity of each frit was 14.7 EZ. The second reagent formula was comprised of 0.0113 g $MgCl_2.6H_20$, 0.0276 g anhydrous $CaCl_2$, 0.51 g lactose, 0.51 g dextran (Pharmachem, Mol. Wt. 17,900), 1.02 g bovine serum albumin (Sigma, fatty acid free), 0.30 g glycerol, 0.187 g 4-aminoantipyrine (Aldrich) (AAP), 0.1486 g cholesterol ester hydrolase (Amano International, specific activity 8.4 EZ/mg) (CEH), 0.086 g cholesterol oxidase (Boehringer-Mannheim, specific activity 6.2 EZ/mg) dissolved in 10.2 mL of 0.25 M MOPSO buffer (pH 7). The solution was then added to 9 g of custom made LC06 frits (Porex Industries; each frit weighed 9 mg, volume 8.5 μL) in a brown bottle and then processed as described for the first reagent frits above. The moisture content and storage conditions were also the

same. Each of the second reagent frits weighed 11.86 mg and were comprised of BSA (0.85 g), dextran (0.425 g), lactose (0.425 g), glycerol (0.255 g), $CaCl_2$ (0.0.23 g), $MgCl_2$ (0.0094 g), AAP (0.156 g), CEH (0.124 g), CO (0.072 g) and MOPSO (0.525 g). The enzyme activities of the second reagent frits were: CEH, 1.04 EZ; CO, 0.445 EZ. Both reagent frits have been found to be stable at room temperature for at least 16 months in terms of their assay performances, in terms of correlation and slope with known cholesterol standards.

b. Reaction Buffer: The reaction buffer (ICMT) which is also the extraction buffer contained the following materials: 0.05 M MOPSO (pH 7) (Sigma), 1% IgePal CO-530 (GAF), 0.2% Triton® X-100 (Bio-Rad) and 0.3% cholic acid (Sigma). The buffer was sterile filtered and was stored at room temperature. The buffer is stable for at least six months.

EXAMPLE 8

PROTOCOL FOR PREPARATION OF A STANDARD CURVE FOR EVALUATION OF THE IMMUNOCAPTURE ASSAY

Fifty or one hundred microliters of the monoclonal antibody-immobilized gels from Examples 4-6 were transferred to Eppendorf micro centrifuge tube. The tube completely filled with 5% BSA in PBS and mixed on a TOMY micro tube mixer (Peninsula Laboratories) at room temperature for one hour in order to block the non-specific binding sites of the plastic tube. The tube then centrifuged on a table-top centrifuge for about one minute and the supernatant carefully aspirated. One hundred microliters of purified lipoprotein fractions diluted in PBS were added to the gels. The gel suspensions were mixed on a TOMY mixer at room temperature for one hour. The gels were then washed twice with about 1 mL of PBS by mixing for one minute, centrifuging for one minute and aspirating the supernatants. ICMT solutions (Example 7(b)) were added to each tube to a final volume of 750 μL. Cholesterol assay reagent frits (one each of #1 and #2 from

Example 7(a)) were added to each tube. The suspensions were mixed on a TOMY mixer for about eight minutes, centrifuged for one minute and the absorbances of the supernatant solutions were read on a DU7400 Spectrophotometer at 515 nm. The concentrations of the gel-bound cholesterol were determined from a cholesterol standard curve. The standard curve was prepared with purified LDL samples having concentrations of 0, 3, 6, 12, 24 and 48 mg/dL following the assay protocol described above (shown in Figure 9).

EXAMPLE 9

PROTOCOL FOR LDL-CHOLESTEROL IMMUNOCAPTURE ASSAY

Four hundred microliters of the monoclonal antibody-immobilized Sepharose 4B gels from Example 4 (about 285 µg of antibody) were transferred to Eppendorf tubes which were previously treated by filling the tubes with 5% BSA in PBS to block all non-specific binding sites. One hundred microliters of individual plasma samples (containing acid-citrate-dextrose or EDTA anticoagulant) were diluted ten-fold in PBS and added to the gels. The gel suspensions were mixed on a TOMY mixer at room temperature for one hour. The gels were then washed twice with about 1 mL of PBS by mixing for one minute, centrifuging for one minute and aspirating the supernatants. ICMT solutions (Example 7(b)) were added to each tube to a final volume of 750 µL. Cholesterol assay reagent frits (one each of #1 and #2 from Example 7(a)) were added to each tube. The suspensions were mixed on a TOMY mixer for about eight minutes, centrifuged for one minute and the absorbances of the supernatant solutions were read on a DU7400 Spectrophotometer at 515 nm. The concentrations of LDL-cholesterol in the plasma samples were determined by multiplying the concentration obtained from the standard curve shown in Figure 9 by 10. The results are shown in Tables 8 and 9, and Figures 10-13 and 16-17.

## EXAMPLE 10

## QUANTITATION OF LDL- AND VLDL-CHOLESTEROL BY ULTRACENTRIFUGATION-POLYANION PRECIPITATION

Plasma samples (7 mL each) were transferred to ultraclear tubes (Beckman, 14 x 95 mm) and then overlayered with 6 mL of d 1.006 g/mL KBr Solution. The samples were centrifuged on a SW40Ti rotor at 40,000 rpm at 4°C for 20 hours. The upper VLDL layers were recovered by a tube-slicing technique. LDL and HDL were recovered in the bottom fraction of each tube. Adequate recovery was verified by comparing the sum of cholesterol in each of the fractions to the total cholesterol of the sample. The cholesterol concentrations of the upper VLDL and lower d >1.006 g/mL (infranet cholesterol) were determined with VISION cholesterol assays (Abbott Laboratories, Abbott Park, Illinois). Assays for HDL-cholesterol concentrations were performed with unfractioned plasma samples using VISION HDL-cholesterol assay (Abbott Laboratories). LDL-cholesterol concentrations were calculated as the difference between in infranet cholesterol and HDL-cholesterol. VLDL-cholesterol concentrations were calculated as the difference between total plasma cholesterol and infranet cholesterol. The results are shown in Table 7.

## EXAMPLE 11

## QUANTITATION OF LDL-CHOLESTEROL BY FRIEDEWALD CALCULATION

LDL-cholesterol concentrations were calculated by using the Friedewald equation: [LDL-cholesterol] = [Total-cholesterol] - [HDL-cholesterol] - [Triglycerides/5]. Total cholesterol, HDL-cholesterol and triglycerides were determined with VISION cholesterol assays and VISION triglyceride assay (Abbott Laboratories). The VISION instrument and each lot of reagent cartridges were calibrated prior to running the plasma specimens. The results are shown in Tables 7 and 9.

EXAMPLE 12

PROTOCOL FOR LDL-CHOLESTEROL ASSAY WITH DRY ANTIBODY-GELS

In order to minimize the time of the assay and also to eliminate the mixing of the test sample with wet gels, the monoclonal antibody 4B5-immobilized Sepharose 4B gel, prepared as described in example 4 of this invention, was used in a dry format. The following is a typical example that was used to demonstrate the proof of principle. To an Eppendorf tube, 400 uL of 4B5-Sepharose 4B gel suspension which contained 100 uL of gel and 280 ug antibody was added and then blocked with 1 mL of 5% alkali-treated casein in PBS at room temperature for one hour. The supernatant was aspirated off and the wet gel was lyophilized at 25 micron vacuum overnight. To the dried powder gel, 100 uL of a ten-fold diluted plasma in PBS was added. The mixture was incubated at room temperature for 10 minutes without any mixing. One mL of PBS was then added to gel to remove the unbound material. After a brief centrifugation and aspiration of the supernatant, the gel was rewashed once again. ICMT solution was then added to the gel to a final volume of 750 uL. After addition of the cholesterol assay reagents, the absorbance of the supernatant solution was read on DU7400 Spectrophotometer at 515 nm. The concentration of the gel-bound cholesterol was obtained from a cholesterol standard curve similar to that shown in Figure 9. The correlation between LDL-cholesterol assays as calculated by the Friedewald equation and by this protocol is illustrated in Figure 20.

EXAMPLE 13

PROTOCOL FOR AN INDIRECT LDL-CHOLESTEROL ASSAY WITH ANTIBODY GEL

This protocol was developed for the following reasons: 1) to demonstrate that 4B5-Sepharose 4B gel specifically and

completely captured the LDL particles from plasma samples used in the direct immunocapture assay; 2) to develop an indirect LDL-cholesterol assay which could be useful in commercial instruments, such as Abbott Vision. The assay format involves the specific capture of LDL particles on 4B5-Sepharose 4B gel and then assay the unbound supernatant containing lipoproteins other than LDL, namely VLDL, IDL, HDL and Lp(a). LDL-cholesterol is then calculated by subtracting from the total cholesterol:

[LDL-chol] = [Total-chol] - [Supernatant-chol].

The following is a typical protocol used in the present invention. To an Eppendorf tube, 400 uL of 4B5-Sepharose which contained 100 uL of gel and 280 ug antibody was added and then blocked with 1 mL of 5% alkali-treated casein in PBS at room temperature for one hour. The supernatant was aspirated off. One hundred microliters of a ten-fold diluted plasma in PBS was added. After mixing at room temperature for ten minutes, 150 uL of PBS was added to the mixture. After a brief centrifugation, ICMT solution was added to the supernatant to a final volume of 750 uL. The cholesterol content in the supernatant was determined as described in example 10. The correlation between LDL-cholesterol assays as calculated by the Friedewald equation and by this indirect method is illustrated in Figure 21. It should be noted that similar results were also obtained using dry 4B5-Sepharose 4B gel.

Lp(a)-CHOLESTEROL SPECIFIC ASSAY

The present invention is further directed to a method for the direct measurement of Lp(a)-cholesterol in plasma, preferably using sandwich immunoassay methodology. A specific binding agent, preferably an antibody and more preferably a monoclonal antibody, specific for Lp(a) is used to capture Lp(a) particles in a plasma sample. The cholesterol

associated with the Lp(a) particles is then measured as described earlier herein. Preferably, the captured Lp(a) particles are separated from the remainder of the sample prior to the cholesterol measurement. To simplify the separation process, the Lp(a) specific binding agent is preferably coupled to a solid phase as described earlier herein.

EXAMPLE 14

PREPARATION OF MONOCLONAL ANTIBODIES SPECIFIC FOR LP(A)

Female BALB/c mice were immunized four times in 2-3 week intervals with 50 µg of apo(a) protein which was emulsified with Ribi adjuvant (Ribi Immunochem Research, Inc., Hamilton, MT). Four days after the last boosing, the mice were sacrificed and the immune spleen cells were fused with myeloma cells SP2/0 according to the procedure reported by Gefter, et al. (1977) Somatic Cell Genet. 3:231. After two to three weeks of hybrid cell growth in microtiter plate wells, tissue culture spent media were collected from hybrid growing wells and tested for Lp(a) binding monoclonal antibodies. The screening procedure was carried out by first incubating the tissue culture spent media on a Lp(a) or Apo(a) coated microtiter plate. Then, after removing the media and washing the wells, horseradish peroxidase labeled goat anti-mouse antibody was incubated in the wells. The wells were again washed and o-phenylenediamine was added to each well for signal development. The microtiter plate was read at 492 nm using a microtiter plate reader. The presence and/or amount of signal development indicated the presence and/or concentration of anti-Lp(a) antibody produced in the tissue culture spent media.

The Lp(a) specific monoclonal antibodies 4D2, 1E1 and 4F2 prepared by the above method were purified on a Protein A-Sepharose 4B column (commercially available from Pharmacia) using 100 mM citrate buffer, pH 6.0, to elute the antibodies from the column. The antibodies did not show any cross-reactivity with LDL, VLDL, IDL and HDL. Also, these

antibodies did not show any significant inhibition of binding to Lp(a) coated microtiter plates with human plasminogen up to 1 mg/mL.

EXAMPLE 15

PREPARATION OF Lp(a) STANDARDS AND CALIBRATORS

Lp(a) concentrations in fresh plasma samples were measured using a commercial ELISA test for Lp(a) (TERUMO Medical Corp., Elkton, MD). Plasma samples with high Lp(a) concentrations were ultracentrifuged for 20 hours at 40,000 rpm at a density of 1.080 g/mL. The upper lipoprotein fraction containing Lp(a), LDL, VLDL and IDL was dialyzed and then the Lp(a) was affinity purified on a Lp(a) specific monoclonal antibody (4F2) Sepharose 4B column using standard procedures well known in the art. The purity of the Lp(a) obtained from the column was determined by polyacrylamide gel electrophoresis under denatured conditions, by SDS-PAGE electrophoresis under reducing conditions and by Western Blot. The protein content of the Lp(a) obtained form the column was measured by Lowry assay and the cholesterol concentration was measured using the Abbott Vision® Cholesterol Assay (commercially available from Abbott Laboratories, Abbott Park, IL). Lp(a) standards having a protein concentration within the range of about 0.3 mg/mL and about 0.6 mg/mL were prepared from the purified Lp(a) by dilution in 20 mM phosphate buffered saline at pH 7.4 or 1% alkali-treated casein in 20 mM phosphate buffered saline at pH 7.4. Calibrators having Lp(a)-cholesterol concentrations of 0, 2.4, 4.85, 9.7, 19.5, 38.9, 77.8, 155.6 and 311 µg/mL were prepared by dilution of the Lp(a) standards in 20 mM phosphate buffered saline at pH 7.4 or 1% alkali-treated casein in 20 mM phosphate buffered saline at pH 7.4. The Lp(a) standards and calibrators were stored at 4°C.

EXAMPLE 16

PREPARATION OF DIGITONIN-PEROXIDASE CONJUGATE

Three parts of a digitonin solution (2.5 mg/mL in water) (Sigma Chemical company, St. Louis, MO) was mixed with one part of a fresh solution of sodium meta-periodate (1.68% w/v in water) at 4°C for one hour and then the mixture was dialyzed against 20 mM phosphate buffered saline (PBS), pH 8.0 at 4°C overnight. One part of a solution of 0.25 M ethylenediamine in 20 mM PBS, pH 8.0, was added to four parts of the dialyzed mixture and the mixture was incubated at 4°C. After 30 minutes and and again after 60 minutes of incubation, 100 µL of a sodium borohydride solution (4 mg/mL in 0.1N NaOH) was added to the mixture for each 30 mg of digitonin in the mixture. The mixture was then incubated for two hours at 4°C. The resulting mixture containing ethylenediamine derivatized digitonin was dialyzed against 0.01 M carbonate buffer, pH 9.5, at 4°C overnight. The final carbonate buffer solution of ethylenediamine derivatized digitonin contained about 1.5 mg digitonin/mL buffer

Twenty-five milligrams of horseradish peroxidase (HRPO) (155 Ku/mg, commercially available from Amano International) were dissolved in 6.25 mL of water and 1.25 mL of freshly prepared 0.2 M sodium meta-periodate was added. After 20 minutes in the dark at room termperature, the reaction was dialyzed against 4 liters of 1 mM acetate buffer, pH 4.5, at 4°C for 4 hours. The oxidized HRPO solution and the ethylenediamine derivatized digitonin solution were mixed and stirred in the dark at room temperature for two hours. Then 400 µL of a sodium borohydride solution (4 mg/mL in water) was added and the reaction was incubated at 4°C. After two hours, the mixture was dialyzed against 20 mM PBS, pH 7.4, at 4°C overnight. One part of 5% w/v aqueous sodium deoxycholate was added to 10 parts of the dialyzed reaction mixture.and fatty acid free bovine serum albumin (BSA) (Sigma Chemical Company) was added to a final concentration of 5 mg/mL. The solution of HRPO-digitonin conjugate was

sterile filtered through a 0.22 micro filter (Coaster Labs) and stored at -20°C.

The binding curves of the HRPO-digitonin conjugate binding to Lp(a)-cholesterol, LDL-cholesterol and VLDL-cholesterol particles were obtained (see Figure 22). Maxisob Nunc Immuno plates were coated by incubating in separate wells 100 µL of a solution containing pure Lp(a), LDL or VLDL having a cholesterol concentration of about 5 µg/mL in 20 mM PBS, at pH 7.4, at 37°C for one hour. The wells were washed five times with 0.05% w/v Tween 20 in 20 mM PBS, at pH 7.4 (PBS-Tween) and then blocked with 200 µL of 5% w/v BSA in 20 mM PBS, at pH 7.4, by incubation at 37°C for one hour. The HRPO-digitonin conjugate solution was serially diluted in the wells with a solution of 5 µg/mL of alkali-treated casein in 20 mM PBS, at pH 7.4 (100µL total volume in each well). After incubation at 37°C for one hour, the wells were washed eight times with PBS-Tween. One hundred microliters of a o-phenylenediamine substrate solution (commercially available from Abbott Laboratories) was added to each well and after five minutes, the reaction was quenched with the addition of 100 µL of 1N sulfuric acid. The absorbance in each well was measured on a Bio-Tek microplate reader at 490 nm.

## EXAMPLE 17

## Lp(a)-CHOLESTEROL ASSAY

The monoclonal antibody 1E1 was diluted in 20 mM PBS, at pH 7.4, to a concentration of 5 µg/mL. One hundred microliters of the 1E1 solution was added to the wells of Maxisob Nunc Immuno plates and the plates were incubated at room temperature on a rotator at 100 rpm for two hours. The plates were washed five times with PBS-Tween solutions and then blocked with 200 µL of 5% w/v BSA in 20 mM PBS, at pH 7.4, by incubation at 37°C for one hour. The plates can be stored at 4°C with plastic sealers at least for ten days prior to use.

Plasma samples were diluted 201-fold with 1% w/v alkali-treated casein in 20 mM PBS, at pH 7.4. One hundred microliters of the diluted samples were added to each well of the 1E1 plates and the plates were incubated at 37°C for one hour. After washing the plates five times with PBS-Tween, 100 µL of HRPO-digitonin conjugate (2 µg/mL in 1% w/v alkali-treated casein in 20 mM PBS at pH 7.4) were added to each well. The plates were incubated at 37°C for one hour and then washed ten times with PBS-Tween. One-hundred microliters of a freshly prepared solution of o-phenylenediamine in citrate buffer (substrate commercially available from Abbott Laboratories) were added to each well and after five minutes, the reaction was quenched with 100 µL of 1N sulfuric acid. The absorbance of each well was measured on a Bio-Tek microplate reader at 490 nm. The Lp(a)-cholesterol concentration was then determined from a standard curve of absorbance versus Lp(a)-cholesterol concentration.

EXAMPLE 18

Lp(a)-CHOLESTEROL CALIBRATION CURVE

Lp(a)-cholesterol standards were prepared from Lp(a) standard solutions as described in Example 15. Calibrators having Lp(a)-cholesterol concentrations of 0, 2.4, 4.85, 9.7, 19.5, 38.9, 77.8, 155.6 and 311 µg/mL were assayed by the method described in Example 17. The concentrations were multiplied by 201 to generate the standard curve because the plasma samples were diluted 201-fold prior to performing the assay. A plot of Lp(a)-cholesterol concentration versus absorbance was prepared from the resulting data. Figure 23 is illustrative of such a plot. The Lp(a)-cholesterol concentration in unknown samples can be determined from the calibration curve. Generally the calibrators and the plasma samples were assayed on the same plate to minimize the effect of variations in the reagents, materials and conditions.

The number and concentration of calibrators can be readily altered depending on the desired accuracy of the results.

EXAMPLE 19

PLASMA SAMPLES

The lipid profiles of 64 plasma samples from individuals without known cardiac problems ("N") and patients with mixed hyperlipidemia ("MHL"), hypocholesterolemia ("HC") and mild hypocholesterolemia ("MHC") were determined by the methods described earlier herein and the results are shown in Table 11. Total cholesterol, HDL-cholesterol and triglyceride concentations were measured using an Abbott Vision® instrument and reagents (commercially available from Abbott Laboratories, Abbott Park, IL).

The Lp(a)-cholesterol concentrations of the samples were determined using a commercial ELISA test for Lp(a) (TERUMO Medical Corp., Elkton, MD), standard calculated values based on the total Lp(a) concentration and the method of the present invention disclosed in Example 17. The results are summarized in Table 12. The results in Table 12 illustrate the good correlation between the TERUMO ELISA method and the method of the present method (correlation coefficient (r) = 0.983; slope - 0.935; and intercept = 0.712) for normal individuals. The TERUMO ELISA method tended to produce erroneous results for the cardiac patient samples, especially those with high concentrations of Lp(a) (>40 mg/dL). The results obtained by the present method had excellent correlation with the Lp(a)-protein assay method for samples from both normal individuals and cardiac patients (correlation coefficient (r) = 0.972; slope = 0.997; intercept = 1.0).

The data in Table 12 appears to indicate that the cholesterol content in Lp(a) particles is not constant for each individual and hence the assumptions behind the calculated values of Lp(a)-cholesterol are erroneous. With the discovery of the present invention, it is now possible to accurately measure the Lp(a)-cholesterol plasma concentration directly

rather than assume that a constant relationship exists for all individuals. The correlation between disease states and Lp(a)-cholesterol concentrations can now be readily established using the present invention. In addition, the effect of proposed treatments of such disease states can now be easily monitored using the present invention.

TABLE 11

LIPID PROFILES OF Lp(a) PLASMA SAMPLES

| Sampl. No. | Total Chol. mg/dL | HDL-Chol. mg/dL | TRIG[1] mg/dL | F.E.[2] LDL-Chol. mg/dL | U.C.[3] LDL-Chol. mg/dL | U.C.[4] VLDL-Chol. mg/dL | Sampl. Type |
|---|---|---|---|---|---|---|---|
| 101 | 253 | 50 | 80 | 187 | 179 | 23 | N |
| 102 | 175 | 70 | 50 | 95 | 92 | 13 | N |
| 103 | 245 | 50 | 140 | 167 | 148 | 47 | N |
| 104 | 167 | 53 | 120 | 90 | 90 | 24 | N |
| 105 | 239 | 76 | 60 | 151 | 142 | 21 | N |
| 106 | 198 | 34 | 110 | 141 | 131 | 33 | N |
| 107 | 177 | 43 | 200 | 94 | 97 | 37 | N |
| 108 | 171 | 32 | 135 | 112 | 104 | 35 | N |
| 109 | 213 | 58 | 75 | 141 | 143 | 12 | N |
| 110 | 224 | 50 | 290 | 108 | 108 | 67 | N |
| 111 | 200 | 50 | 125 | 124 | 111 | 39 | N |
| 112 | 194 | 68 | 75 | 111 | 106 | 20 | N |
| 113 | 181 | 55 | 50 | 117 | 114 | 12 | N |
| 114 | 194 | 62 | 60 | 121 | 126 | 12 | N |
| 115 | 240 | 52 | 105 | 167 | 165 | 23 | N |
| 116 | 155 | 57 | 60 | 85 | 84 | 12* | N |
| 117 | 179 | 43 | 72 | 122 | 118 | 14* | N |
| 118 | 152 | 68 | 35 | 77 | 74 | 10 | N |
| 119 | 193 | 34 | 175 | 123 | 119 | 40 | N |
| 120 | 251 | 41 | 144 | 182 | 185 | 29* | N |
| 121 | 168 | 43 | 106 | 104 | 110 | 21* | N |
| 122 | 283 | 42 | 530 | | 171 | 71 | N |
| 123 | 172 | 55 | 67 | 104 | 101 | 13* | N |
| 124 | 203 | 50 | 118 | 130 | 132 | 24* | N |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 125 | 183 | 43 | 111 | 118 | 120 | 22* | N |
| 126 | 134 | 56 | 55 | 67 | 66 | 12 | N |
| 127 | 156 | 50 | 54 | 96 | 92 | 11* | N |
| 128 | 289 | 50 | 240 | 191 | 176 | 63 | N |
| 129 | 143 | 50 | 49 | 83 | 85 | 10* | N |
| 130 | 160 | 61 | 52 | 89 | 87 | 10* | N |
| 131 | 201 | 41 | 82 | 144 | 138 | 16* | N |
| 132 | 180 | 30 | 198 | 110 | 115 | 40* | N |
| 133 | 186 | 53 | 83 | 116 | 116 | 17* | N |
| 134 | 220 | 52 | 93 | 149 | 145 | 19* | N |
| 135 | 227 | 35 | 243 | 144 | 140 | 49* | N |
| 136 | 167 | 55 | 45 | 102 | 98 | 9* | N |
| 137 | 239 | 35 | 489 | | 125 | 79 | MHL |
| 138 | 217 | 48 | 181 | 133 | 145 | 24 | MHL |
| 139 | 250 | 26 | 289 | 166 | 160 | 64 | MHL |
| 140 | 183 | 32 | 292 | 93 | 109 | 42 | N |
| 141 | 140 | 25 | 226 | 70 | 90 | 17 | HC |
| 142 | 257 | 50 | 84 | 190 | 163 | 14 | HC |
| 143 | 199 | 60 | 121 | 115 | 115 | 24* | N |
| 144 | 222 | 51 | 73 | 156 | 156 | 15* | MHC |
| 145 | 226 | 48 | 127 | 153 | 153 | 25* | MHC |
| 146 | 192 | 32 | 168 | 126 | 126 | 34* | N |
| 147 | 215 | 36 | 79 | 163 | 163 | 16* | MHC |
| 148 | 267 | 35 | 138 | 204 | 204 | 28* | HC |
| 149 | 225 | 20 | 464 | | | | MHL |
| 150 | 186 | 29 | 531 | | 87 | 70 | N |
| 151 | 245 | 50 | 136 | 168 | 173 | 22 | HC |
| 152 | 249 | 68 | 79 | 165 | 169 | 12 | HC |
| 153 | 236 | 37 | 256 | 148 | 138 | 61 | MHL |
| 154 | 160 | 26 | 111 | 112 | 109 | 13 | N |
| 155 | 141 | 49 | 131 | 66 | 81 | 11 | HC |
| 156 | 197 | 35 | 214 | 119 | 137 | 25 | N |
| 157 | 236 | 38 | 381 | 122 | 136 | 62 | MHL |
| 158 | 235 | 77 | 190 | 120 | 120 | 38* | MHL |
| 159 | 153 | 48 | 123 | 80 | 80 | 25* | N |
| 160 | 154 | 45 | 118 | 85 | 85 | 24* | N |
| 161 | 169 | 64 | 61 | 93 | 93 | 12* | N |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 162 | 180 | 47 | 132 | 107 | 120 | 13 | N |
| 163 | 177 | 40 | 61 | 125 | 127 | 10 | N |
| 164 | 200 | 58 | 87 | 125 | 136 | 6 | N |

1 TRIG = triglyceride concentration.
2 F.E. = Friedewald Equation:
[LDL-Chol] = [Total Chol] - [HDL-Chol] - [TRIG/5].
3 U.C. = Ultracentrifuge ß-quantitation:
[LDL-Chol] = [d >1.006 Infranate-Chol] - [HDL-Chol].
4 U.C. = Ultracentrifuge ß-quantitation:
[VLDL-Chol] = [Total Chol] - [d >1.006 Infranate-Chol].
* Calculated by dividing the triglyceride concentration by 5.

TABLE 12

Lp(a)-CHOLESTEROL LEVELS OF PLASMA SAMPLES

| Sampl. No. | TERUMO[1] Lp(a) mg/dL | TERUMO[2] Lp(a)-Chol. mg/dL | EIA[3] Lp(a)-Chol. mg/dL | ELISA[4] Lp(a) mg/dL | ELISA[2] Lp(a)-Chol. mg/dL | Sampl. Type |
|---|---|---|---|---|---|---|
| 101 | 19.6 | 5.88 | 5.6 | | | N |
| 102 | 7.9 | 2.1 | 3.8 | | | N |
| 103 | 80.6 | 24.2 | 24.5 | | | N |
| 104 | 15.14 | 4.54 | 5.2 | | | N |
| 105 | 35.88 | 10.76 | 8.8 | | | N |
| 106 | 2.68 | 0.8 | 0.66 | | | N |
| 107 | 1.16 | 0.3 | 0.2 | | | N |
| 108 | 10.2 | 3.1 | 4.4 | | | N |
| 109 | 12.72 | 3.8 | 2.96 | | | N |
| 110 | 18.36 | 5.5 | 5.67 | | | N |
| 111 | 7.5 | 2.3 | 3.2 | | | N |
| 112 | 14.64 | 4.4 | 4.1 | | | N |
| 113 | 84.2 | 25.2 | 21.5 | | | N |
| 114 | 65.3 | 19.6 | 21.5 | | | N |
| 115 | 25.69 | 7.7 | 5.6 | | | N |
| 116 | 12.5 | 3.74 | 4.69 | | | N |
| 117 | 4.86 | 1.46 | 3.76 | | | N |
| 118 | 4.23 | 1.27 | 1.38 | | | N |

| | | | | | | |
|---|---|---|---|---|---|---|
| 119 | 9.2 | 2.8 | 4.8 | | | N |
| 120 | 65.3 | 19.6 | 17.7 | | | N |
| 121 | 14.7 | 4.4 | 4.4 | | | N |
| 122 | 0.7 | 0.2 | 0.12 | | | N |
| 123 | 35.7 | 10.7 | 12.5 | | | N |
| 124 | 0.78 | 0.2 | 0.1 | | | N |
| 125 | 62.6 | 18.8 | 19.88 | | | N |
| 126 | 4.6 | 1.4 | 2.1 | | | N |
| 127 | 1.8 | 0.5 | 0.34 | | | N |
| 128 | 3.2 | 0.97 | 0.6 | | | N |
| 129 | 6.7 | 2.0 | 2.9 | | | N |
| 130 | 3.3 | 1.0 | 2.5 | | | N |
| 131 | 13.3 | 4.0 | 5.9 | | | N |
| 132 | 10.0 | 3.0 | 4.6 | | | N |
| 133 | 6.7 | 2.0 | 3.1 | | | N |
| 134 | 0.7 | 0.2 | 0.13 | | | N |
| 135 | 1.34 | 0.4 | 0.57 | | | N |
| 136 | 6.7 | 2.0 | 3.7 | | | N |
| 137 | 1.6 | 0.48 | 0.66 | 2.48 | 0.74 | MHL |
| 138 | 10.53 | 3.2 | 5.23 | 7.58 | 2.3 | MHL |
| 139 | 36.2 | 10.86 | 5.2 | 18.95 | 5.68 | MHL |
| 140 | 3.16 | 0.95 | 0.45 | 2.65 | 0.8 | N |
| 141* | 65.68 | 19.7 | 19.7 | 61.5 | 18.4 | HC |
| 142* | 67.36 | 20.2 | 33.6 | 94.3 | 28.3 | HC |
| 143* | 88.4 | 26.5 | 22.4 | 82.94 | 24.88 | N |
| 144* | 46.73 | 14.02 | 13.8 | 35.36 | 10.61 | MHC |
| 145 | 17.68 | 5.3 | 7.59 | 11.37 | 3.41 | MHC |
| 146 | 12.2 | 3.66 | 3.32 | 5.47 | 1.64 | N |
| 147* | 60.6 | 18.19 | 17.56 | 39.57 | 11.87 | MHC |
| 148 | 22.73 | 6.82 | 8.46 | 13.47 | 4.0 | HC |
| 149 | 28.63 | 8.59 | 4.5 | 15.58 | 4.7 | MHL |
| 150 | 7.36 | 2.21 | 0.84 | 5.47 | 1.64 | N |
| 151* | 78.3 | 23.49 | 14.7 | 48.84 | 14.65 | HC |
| 152* | 78.3 | 23.49 | 13.74 | 47.99 | 14.4 | HC |
| 153 | 18.5 | 5.5 | 4.05 | 11.79 | 3.54 | MHL |
| 154 | 55.57 | 16.67 | 9.97 | 36.2 | 10.86 | N |
| 155 | 0.0 | 0.0 | 0.16 | 0.37 | 0.11 | HC |

| | | | | | | |
|---|---|---|---|---|---|---|
| 156 | 11.58 | 3.47 | 3.4 | 5.05 | 1.52 | N |
| 157 | 2.53 | 0.76 | 0.86 | 1.98 | 0.59 | MHL |
| 158 | 12.63 | 3.79 | 3.67 | 8.84 | 2.65 | MHL |
| 159* | 72.0 | 21.6 | 22.26 | 81.25 | 24.38 | N |
| 160* | 67.8 | 20.33 | 20.78 | 66.1 | 19.8 | N |
| 161 | 1.05 | 0.32 | 0.21 | 0.93 | 0.28 | N |
| 162 | 20.0 | 6.0 | 3.4 | 6.74 | 2.0 | N |
| 163 | 20.63 | 6.19 | 3.75 | 10.94 | 3.28 | N |
| 164 | 5.26 | 1.59 | 3.1 | 8.4 | 2.5 | N |

[1] Total Lp(a) measured by TERUMO ELISA test.

[2] Lp(a)-Cholesterol calculated from Total Lp(a) by multiplying Total Lp(a) by 0.3.

[3] Lp(a)-Cholesterol measured directly by EIA according to Example 16.

[4] Total Lp(a) calculated by multiplying the Lp(a)-protein concentration [measured by an ELISA method at the University of Chicago (Dr. A. Scanu)] by 4.21.

* Samples diluted two-fold prior to assay.

## SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: ABBOTT LABORATORIES

(ii) TITLE OF INVENTION: IMMUNOCAPTURE ASSAY FOR DIRECT QUANTIUTATION OF SPECIFIC LIPOPROTEIN CHOLESTEROL LEVELS

(iii) NUMBER OF SEQUENCES: 2

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: ABBOTT LABORATORIES D-377 AP-6D
(B) STREET: ONE ABBOTT PARK ROAD
(C) CITY: ABBOTT PARK

(D) STATE: ILLINOIS
(E) COUNTRY: UNITED STATES OF AMERICA
(F) ZIP: 60064-3500

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/968,619
(B) FILING DATE: 29-OCT-1992

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/847,502
(B) FILING DATE: 06-MAR-1992

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: UNGEMACH, FRANK S
(B) REGISTRATRION NUMBER: 34449
(C) REFERENCE/DOCKET NUMBER: 5144.PC.O1

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 708-937-8360
(B) TELEFAX: 708-938-2623

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Glu Phe His Met Lys Val Lys His Leu Ile Asp Ser Leu
1 5 10
Ile Asp Phe Leu Asn Phe Pro Arg Phe Gln Phe Pro Gly
15 20 25
Lys Pro Gly Ile Tyr Thr Arg Glu Glu Leu
30 35

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Ser Met Pro Ser Phe Ser Ile Leu Gly Ser Asp Val Arg
1 5 10
Val Pro Ser Tyr Thr Leu Ile Leu Pro Ser Leu Glu Leu
15 20 25
Pro Val Leu His Val Pro Arg Asn Lys
30 35

The embodiments described and the alternative embodiments presented are intended as examples rather than as limitations. Thus, the description of the invention is not intended to limit the invention to the particular embodiments disclosed, but it is intended to encompass all equivalents and subject matter within the spirit and scope of the invention as described above and as set forth in the following claims.

WHAT IS CLAIMED IS:

1. A method for determining the amount of cholesterol associated with a specific lipoprotein in a sample comprising:

a. mixing a sample and a lipoprotein specific binding agent such that binding-agent-lipoprotein complexes are formed; and

b. determining the amount of cholesterol bound to said binding-agent-lipoprotein complexes.

2. The method of claim 1 wherein said lipoprotein specific binding agent is coupled to a solid support.

3. The method of claim 2 further comprising the step of separating the solid support from the sample before determining the amount of cholesterol bound to said binding-agent-lipoprotein complexes.

4. The method of claim 3 wherein said determination comprises releasing said cholesterol bound to said binding-agent-lipoprotein complexes and measuring the amount of cholesterol released.

5. The method of claim 3 wherein said determination comprises mixing said binding-agent-lipoprotein complexes with a cholesterol specific binding agent coupled to a detectable label such that a second complex is formed and determining the amount of label bound to said second complex.

6. The method of claim 2 wherein the solid support is selected from the group consisting of nitrocellulose, latex, nylon and polystyrene.

7. The method of claim 1 wherein the solid support is selected from the group consisting of beads, particles, magnetic particles, and glass fiber.

8. The method of claim 1 further comprising the step of separating said binding-agent-lipoprotein complexes from the sample before determining the amount of cholesterol bound to said binding-agent-lipoprotein complexes.

9. The method of claim 8 wherein

a. said lipoprotein specific binding agent is conjugated to a first charged substance; and

b. said separation comprises:

i. contacting said binding-agent-lipoprotein complexes with an insoluble solid phase material which is oppositely charged with respect to said first charged substance, such that said solid phase material attracts and attaches to said first charged substance; and

ii. separating said solid phase material and said sample.

10. The method of claim 9 wherein said charged substances are anionic and cationic monomers or polymers.

11. The method of claim 8 wherein said determination comprises releasing said cholesterol bound to said binding-agent-lipoprotein complexes and measuring the amount of cholesterol released.

12. The method of claim 8 wherein said determination comprises mixing said binding-agent-lipoprotein complexes with a cholesterol specific binding agent coupled to a detectable label such that a second complex is formed and determining the amount of label bound to said second complex.

13. The method of claim 12 wherein said cholesterol specific binding agent coupled to a detectable label is added and said second complex is formed prior to said separation step.

14. The method of claim 1 wherein said specific lipoprotein is selected from the group consisting of LDL, HDL, VLDL, IDL and Lp(a).

15. The method of claim 1 wherein said lipoprotein specific binding agent is a monoclonal or a polyclonal antibody that specifically binds to a lipoprotein selected from the group consisting of LDL, HDL, VLDL, IDL and Lp(a).

16. A method for determining the amount of LDL-cholesterol in a sample comprising:

a. mixing a sample and an LDL specific binding agent coupled to a solid support; and

b. determining the amount of LDL-cholesterol bound to said solid support.

17. The method of claim 16 further comprising the step of separating the solid support from the sample before

determining the amount of LDL-cholesterol bound to said solid support.

18. The method of claim 16 wherein the LDL specific binding agent is an antibody that binds to substantially all LDL, to VLDL at less than about 20% of LDL binding, to IDL at less than about 20% of LDL binding, to Lp(a) at less than about 5% of LDL binding, and to HDL at less than about 2% of LDL binding.

19. The method of claim 16 wherein said solid support is selected from the group consisting of nitrocellulose, latex, nylon and polystyrene.

20. A method for determining the amount of LDL in a sample comprising:

a. mixing a sample and an LDL specific binding agent coupled to a solid support; and

b. determining the amount of LDL bound to said solid support.

21. The method of claim 20 further comprising the step of separating the solid support from the sample before determining the amount of LDL bound to said solid support.

22. The method of claim 20 wherein the LDL specific binding agent is a monoclonal or a polyclonal antibody or fragment thereof that binds to substantially all LDL, to VLDL at less than about 20% of LDL binding, to IDL at less than about 20% of LDL binding, to Lp(a) at less than about 5% of LDL binding, and to HDL at less than about 2% of LDL binding.

23. The method of claim 20 wherein said solid support is selected from the group consisting of nitrocellulose, latex, nylon and polystyrene.

24. An antibody specific for LDL and useful in an LDL specific immunoassay produced by immunization of a mammal with a fragment of LDL containing the T2 region of LDL or subfragment of the T2 region of LDL, where said antibody binds to substantially all LDL, to VLDL at less than about 20% of LDL binding, to IDL at less than about 20% of LDL binding, to Lp(a) at less than about 5% of LDL binding, and to HDL at less than about 2% of LDL binding.

25. A monoclonal antibody specific for LDL prepared by the method comprising the steps of:

a. immunizing a mouse or a rat with a fragment of LDL containing the T2 region of LDL or subfragment of the T2 region of LDL;

b. making a suspension of the mouse or rat spleen cells;

c. fusing the spleen cells with mouse or rat myeloma cells in the presence of a fusion promoter;

d. culturing the fused cells;

e. determining the presence of anti-LDL antibody in the culture media;

f. cloning a hybridoma producing antibody that binds to substantially all LDL, to VLDL at less than about 20% of LDL binding, to IDL at less than about 20% of LDL binding, to Lp(a) at less than about 5% of LDL binding, and to HDL at less than about 2% of LDL binding; and

g. obtaining the antibody from said hybridoma.

26. A method for determining the amount of Lp(a)-cholesterol in a sample comprising:

a. mixing a sample and an Lp(a) specific binding agent coupled to a solid support;

b. separating the solid support from the sample; and

c. determining the amount of cholesterol bound to said solid support.

27. The method of claim 26 wherein the Lp(a) specific binding agent is a monoclonal or a polyclonal antibody or fragment thereof.

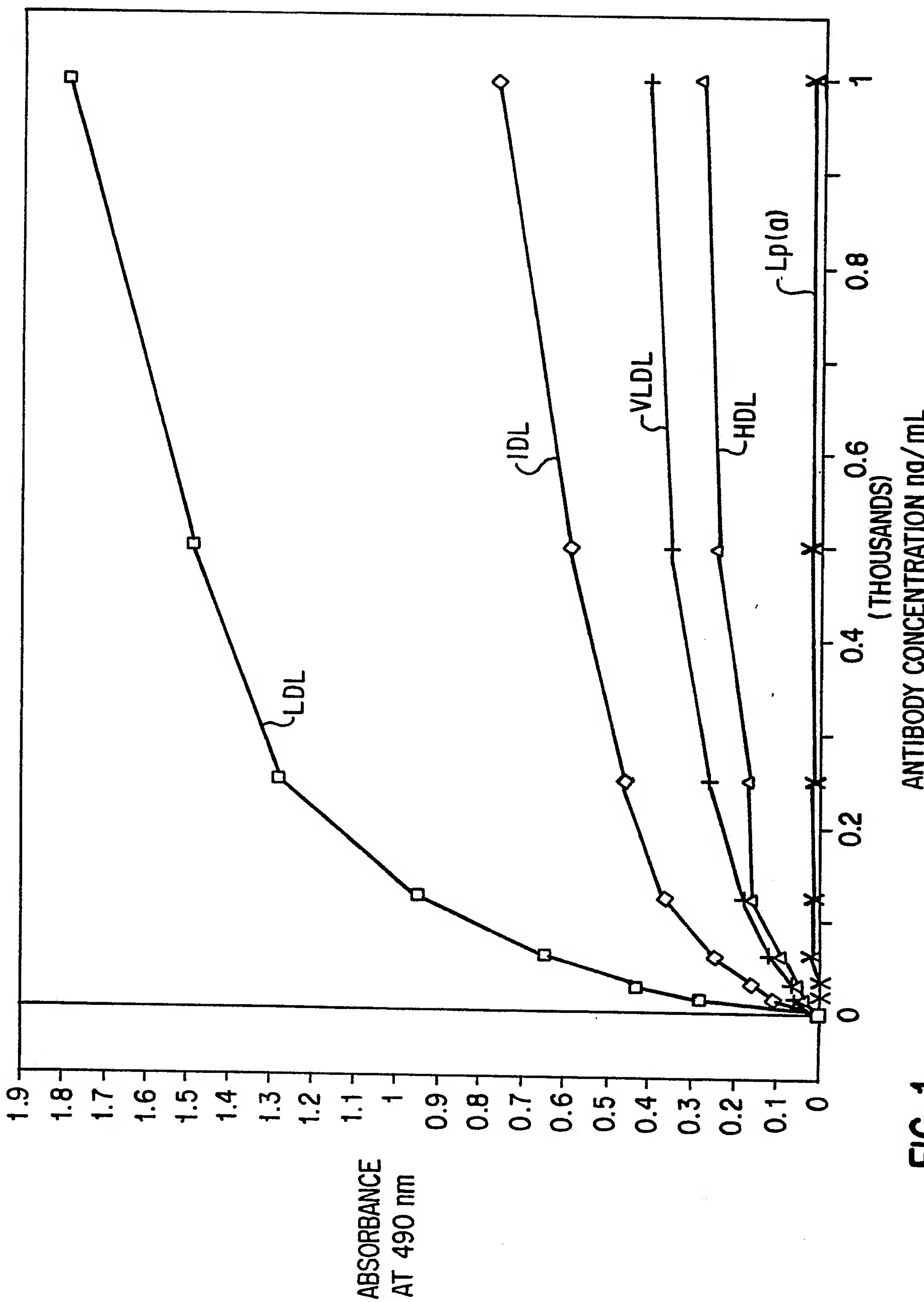

ABSORBANCE AT 490 nm
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
LDL
IDL
VLDL
HDL
Lp(a)
0
0.2
0.4
0.6
0.8
1
(THOUSANDS)
ANTIBODY CONCENTRATION ng/mL

FIG. 1

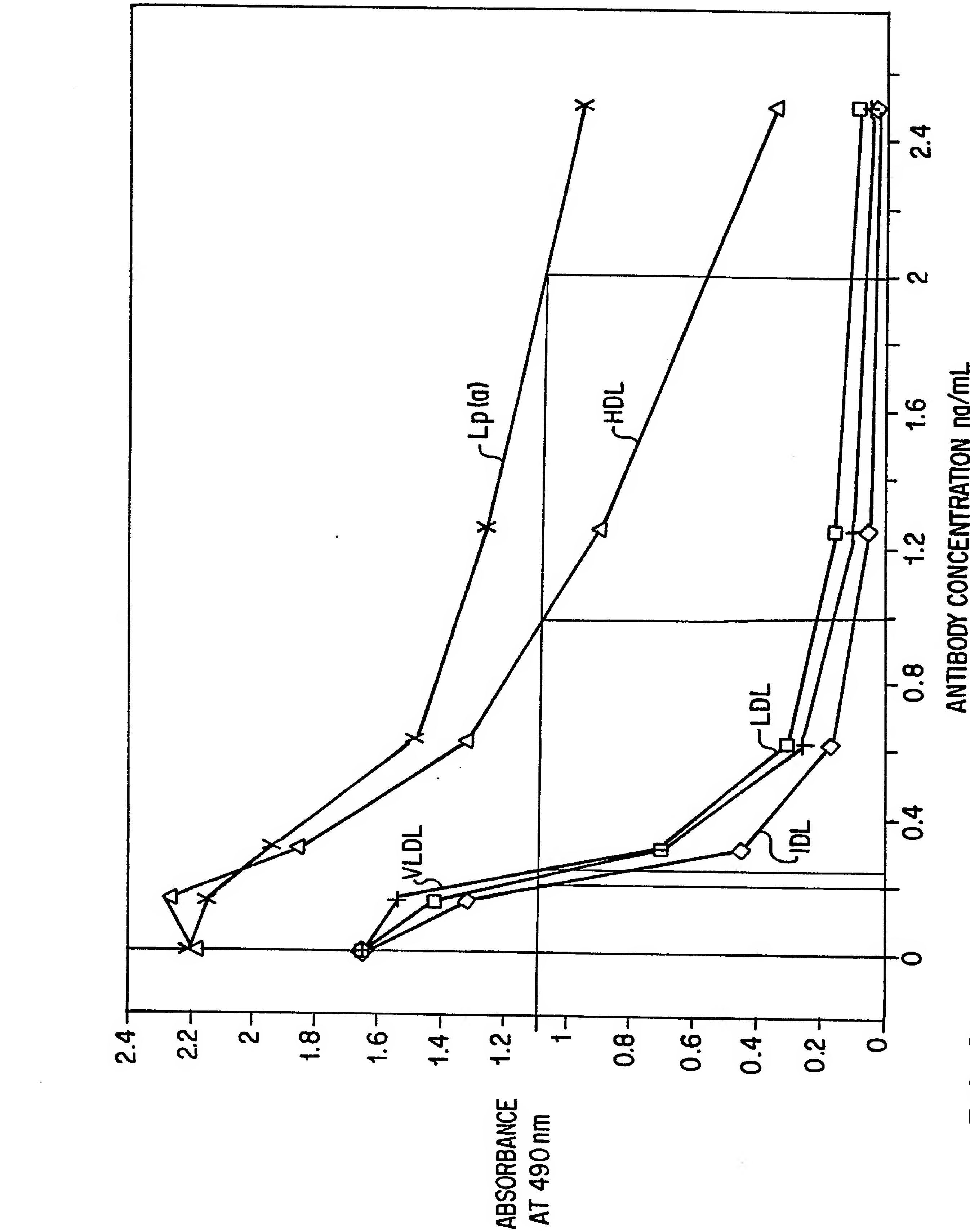

Lp(a)
HDL
LDL
VLDL
IDL
ABSORBANCE AT 490 nm
ANTIBODY CONCENTRATION ng/mL
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
0.4
0.8
1.2
1.6
2
2.4

FIG. 2

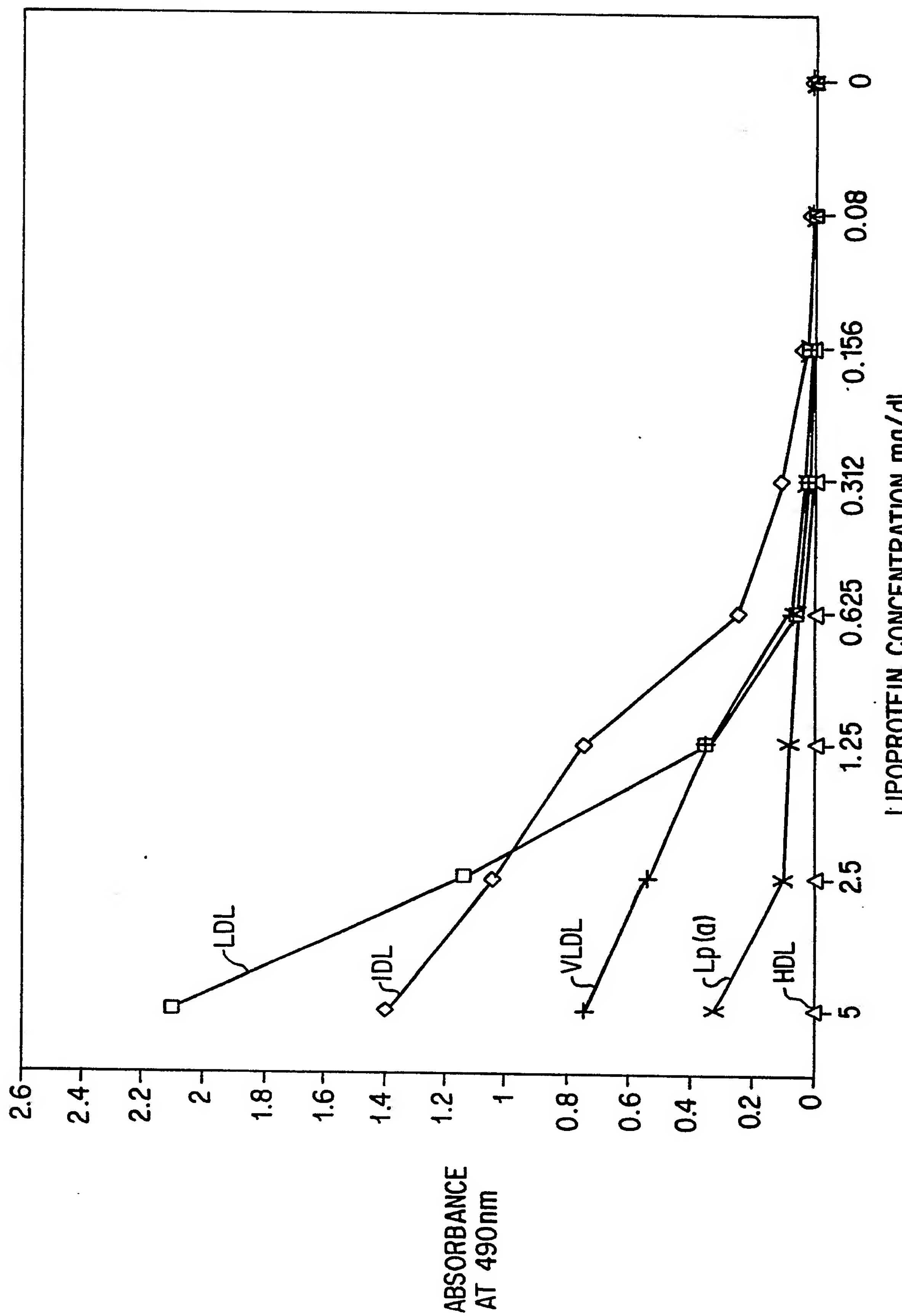

ABSORBANCE AT 490nm
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
5
2.5
1.25
0.625
0.312
0.156
0.08
0
LIPOPROTEIN CONCENTRATION mg/dL
LDL
IDL
VLDL
Lp(a)
HDL

FIG. 3

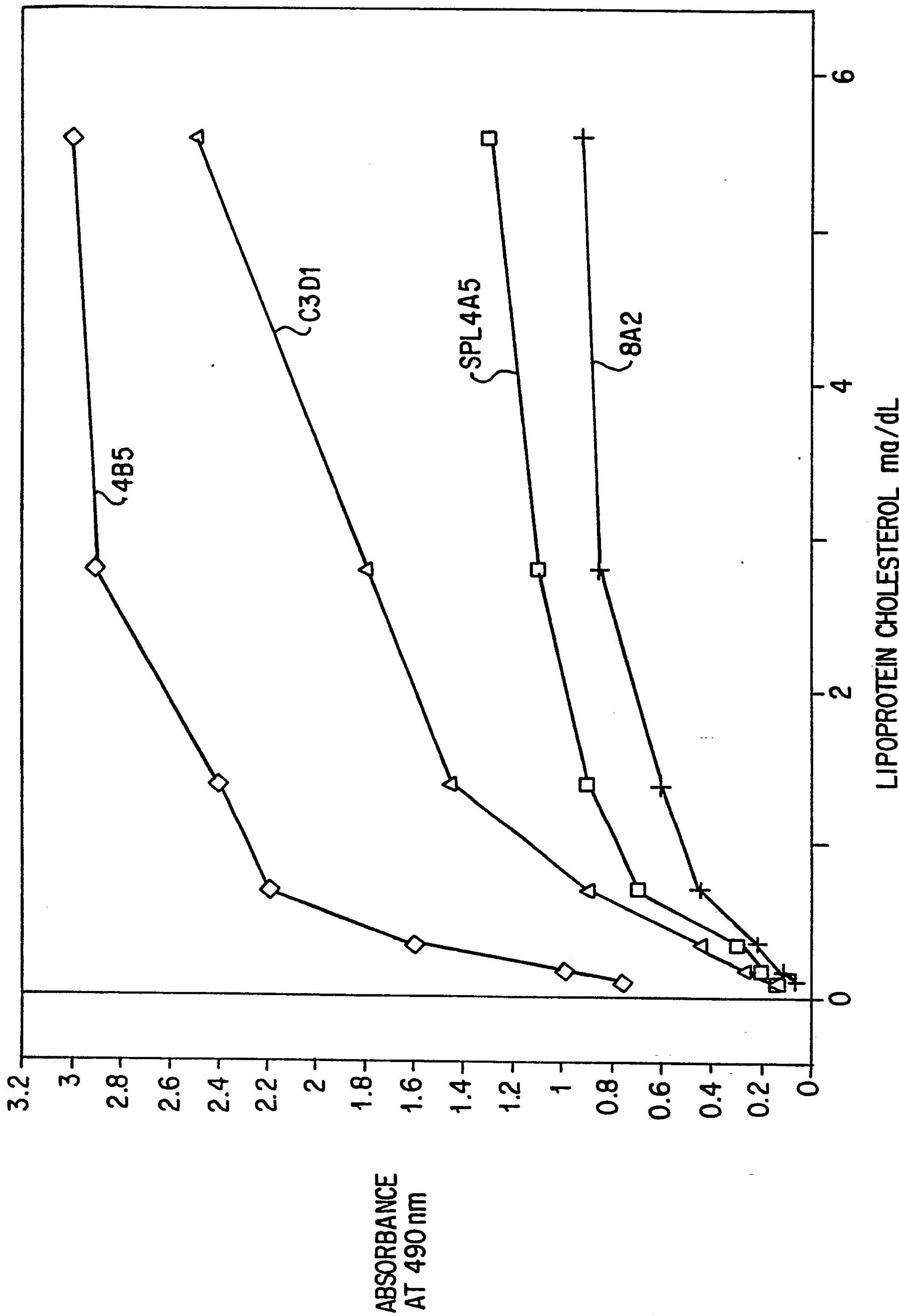
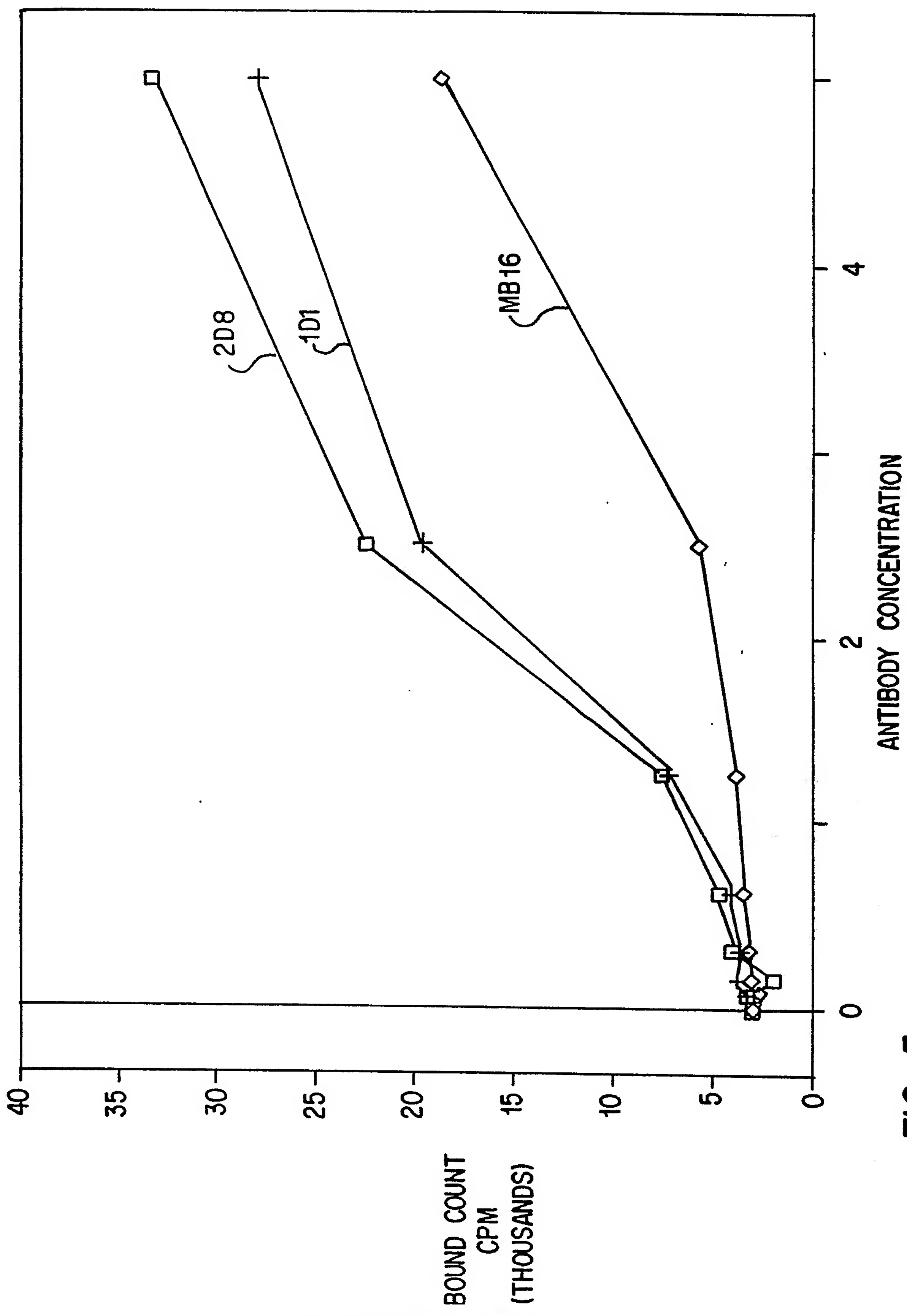

ABSORBANCE AT 490 nm
LIPOPROTEIN CHOLESTEROL mg/dL
4B5
C3D1
SPL4A5
8A2
3.2
3
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6

FIG. 4



2D8
1D1
MB16
ANTIBODY CONCENTRATION
0
2
4
BOUND COUNT
CPM
(THOUSANDS)
0
5
10
15
20
25
30
35
40


FIG. 5

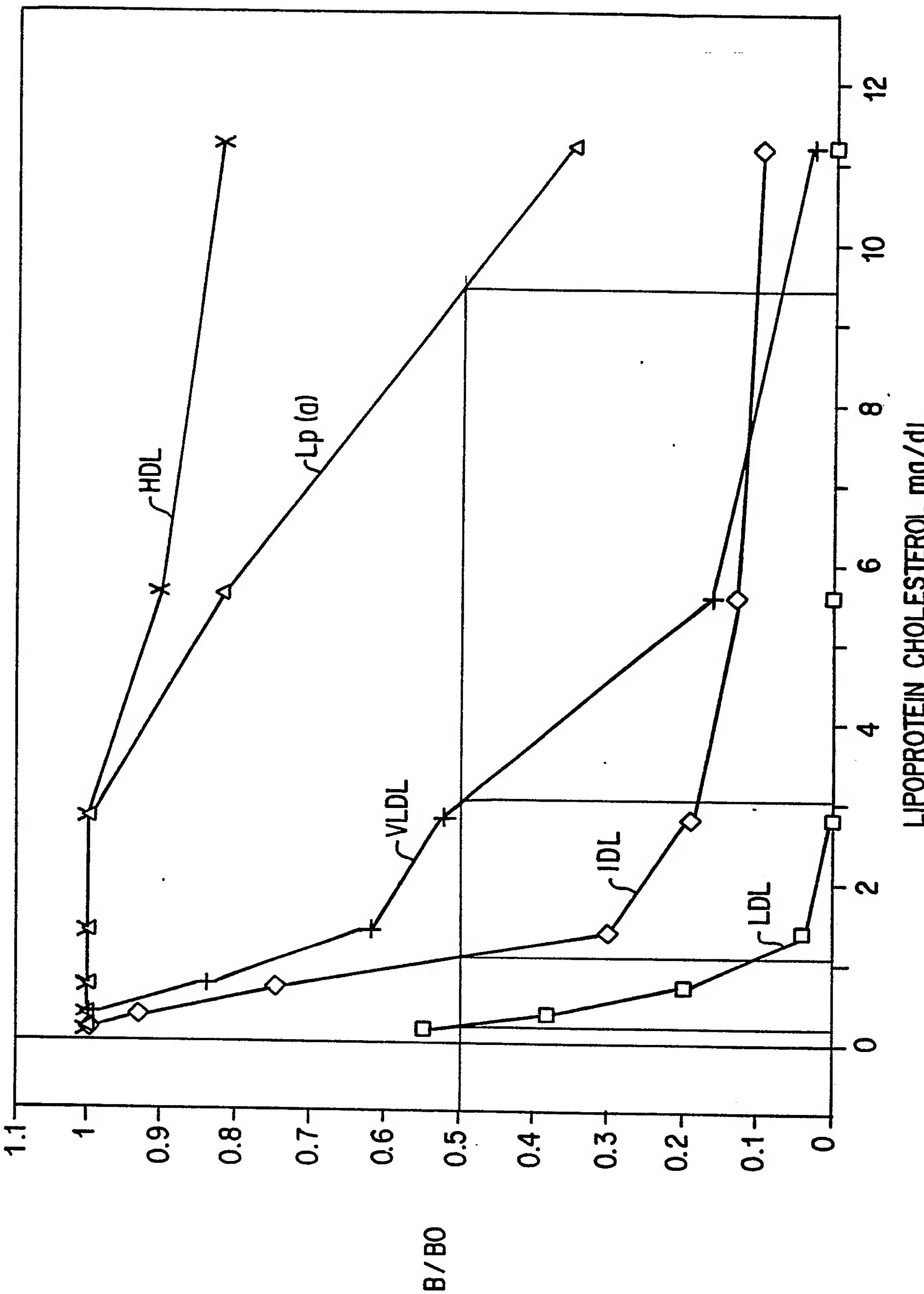

HDL
Lp (a)
VLDL
IDL
LDL
LIPOPROTEIN CHOLESTEROL mg/dL
B/BO
0
2
4
6
8
10
12
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

FIG. 6

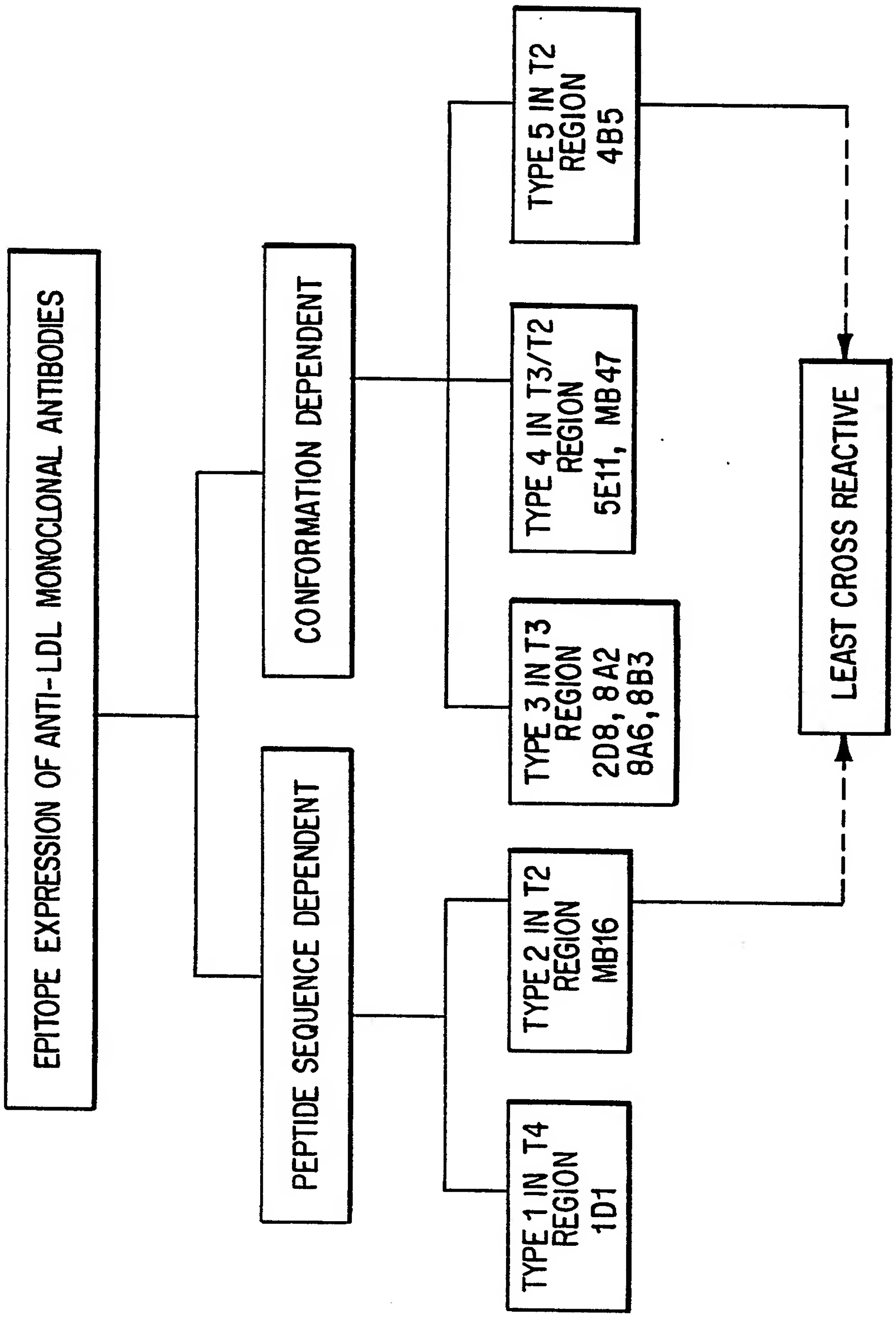
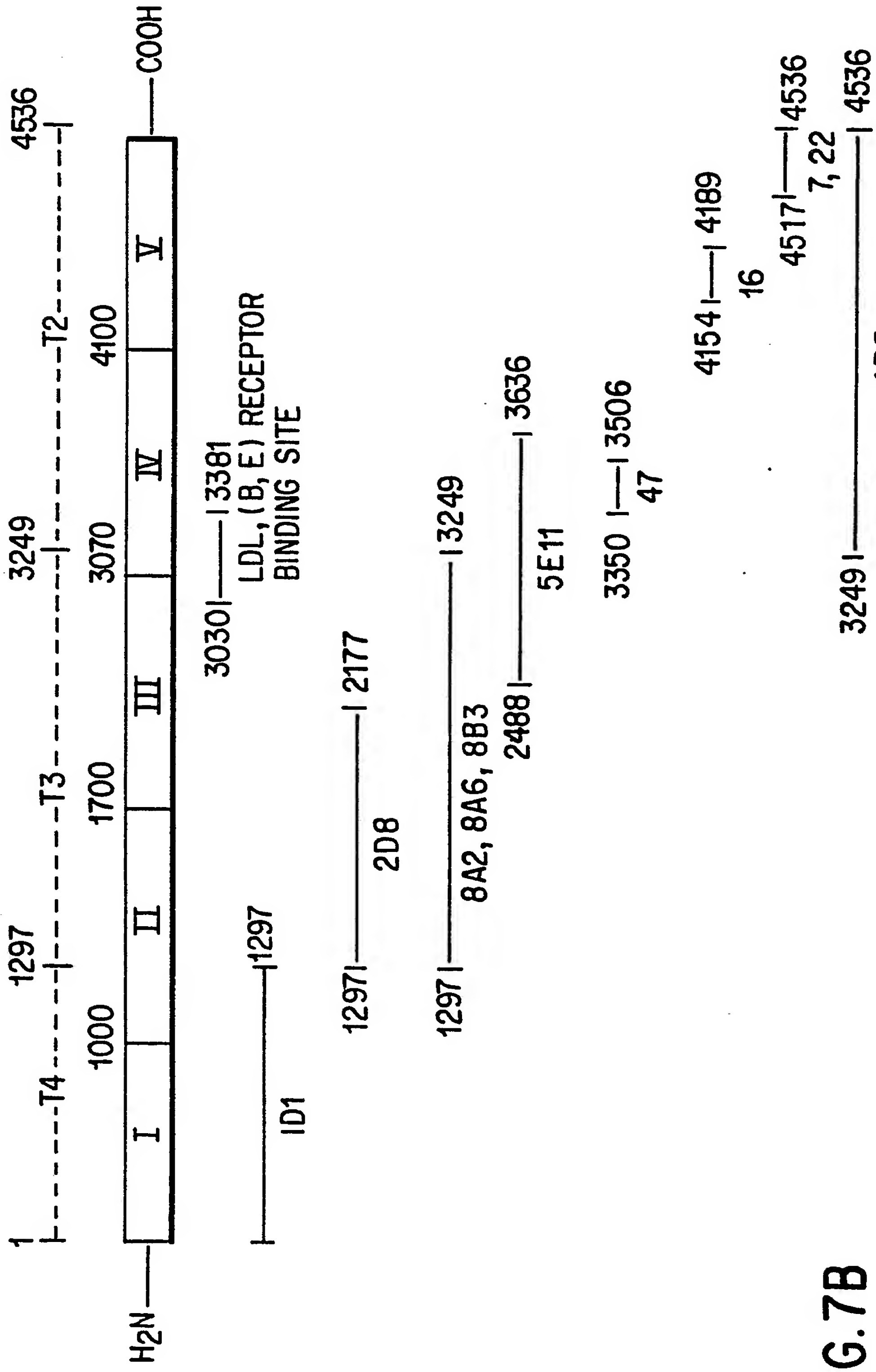

EPITOPE EXPRESSION OF ANTI-LDL MONOCLONAL ANTIBODIES
PEPTIDE SEQUENCE DEPENDENT
CONFORMATION DEPENDENT
TYPE 1 IN T4 REGION 1D1
TYPE 2 IN T2 REGION MB16
TYPE 3 IN T3 REGION 2D8, 8A2 8A6, 8B3
TYPE 4 IN T3/T2 REGION 5E11, MB47
TYPE 5 IN T2 REGION 4B5
LEAST CROSS REACTIVE

FIG. 7A



1
T4
1297
T3
3249
T2
4536
H2N
I
II
III
IV
V
COOH
1000
1700
3070
4100
ID1
1297
3030
3381
LDL, (B, E) RECEPTOR
BINDING SITE
1297
2177
2D8
1297
3249
8A2, 8A6, 8B3
2488
3636
5E11
3350
3506
47
4154
4189
16
4517
4536
7, 22
3249
4536
4B5


FIG.7B

RESIDUE 4154-4189 OF ApoB100 T2 FRAGMENT
(SEQ ID NO 1)

```
Glu Phe His Met Lys Val Lys His Leu Ile Asp Ser Leu Ile Asp
    4155                4160                4165
Phe Leu Asn Phe Pro Arg Phe Gln Phe Pro Gly Lys Pro Gly Ile
    4170                4175                4180
Tyr Thr Arg Glu Glu Leu
    4185
```

FIG. 8A

LDL B RECEPTOR RESIDUE 3252-3286
(SEQ ID NO 2)

```
Ser Met Pro Ser Phe Ser Ile Leu Gly Ser Asp Val Arg Val Pro
            3255                3260                3265
Ser Tyr Thr Leu Ile Leu Pro Ser Leu Glu Leu Pro Val Leu His
            3270                3275                3280
Val Pro Arg Asn Lys
            3285
```

FIG. 8B

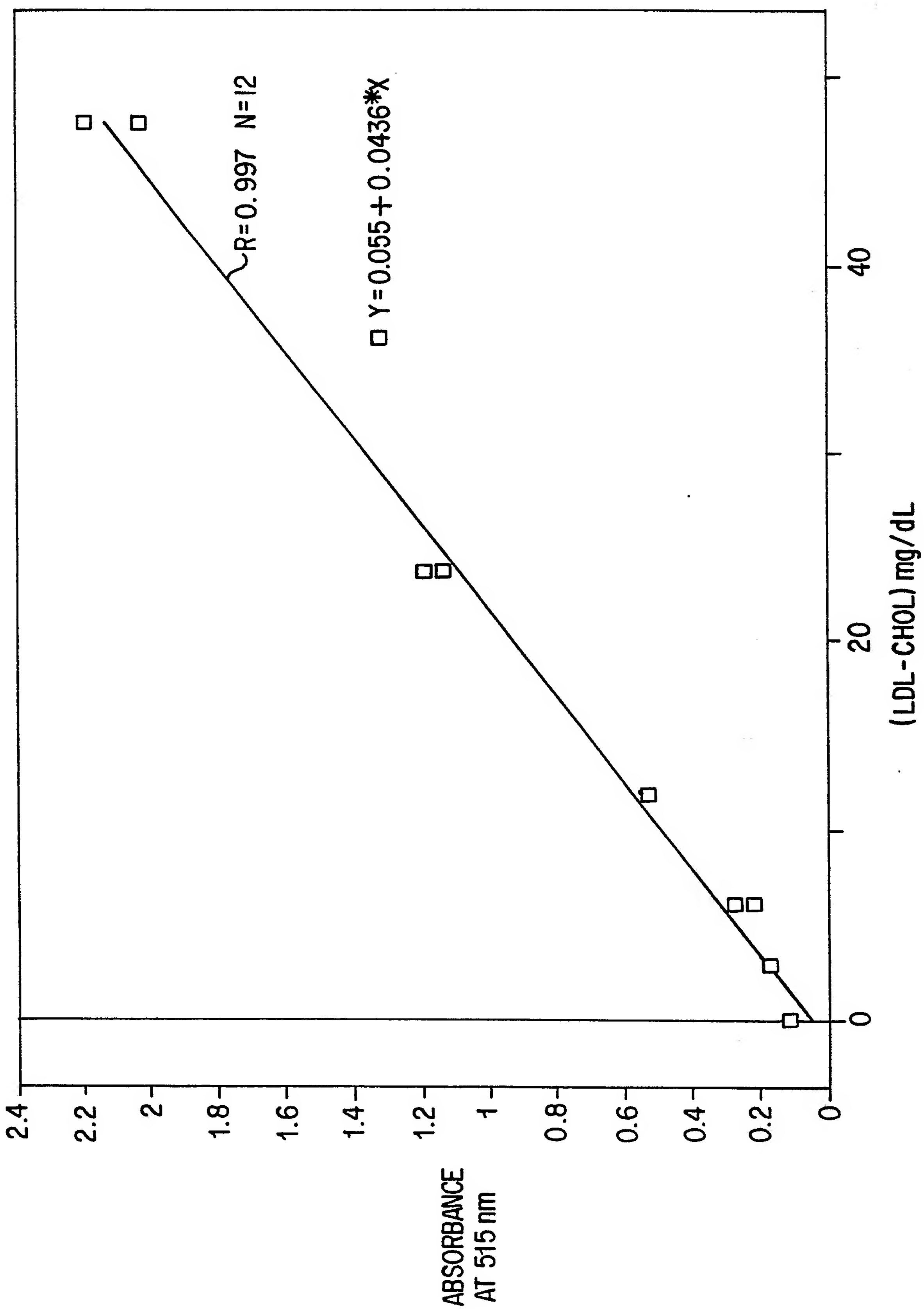

R=0.997 N=12
Y=0.055+0.0436*X
ABSORBANCE
AT 515 nm
(LDL-CHOL) mg/dL
0
20
40
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4

FIG. 9

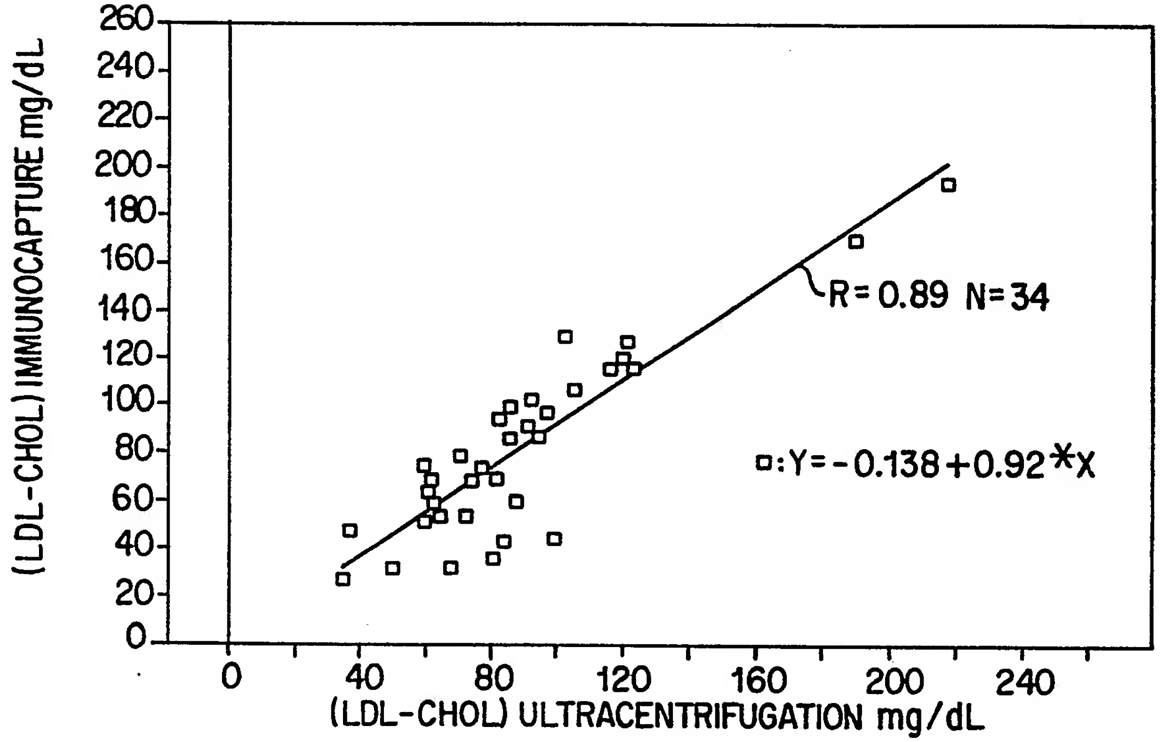



FIG.10A

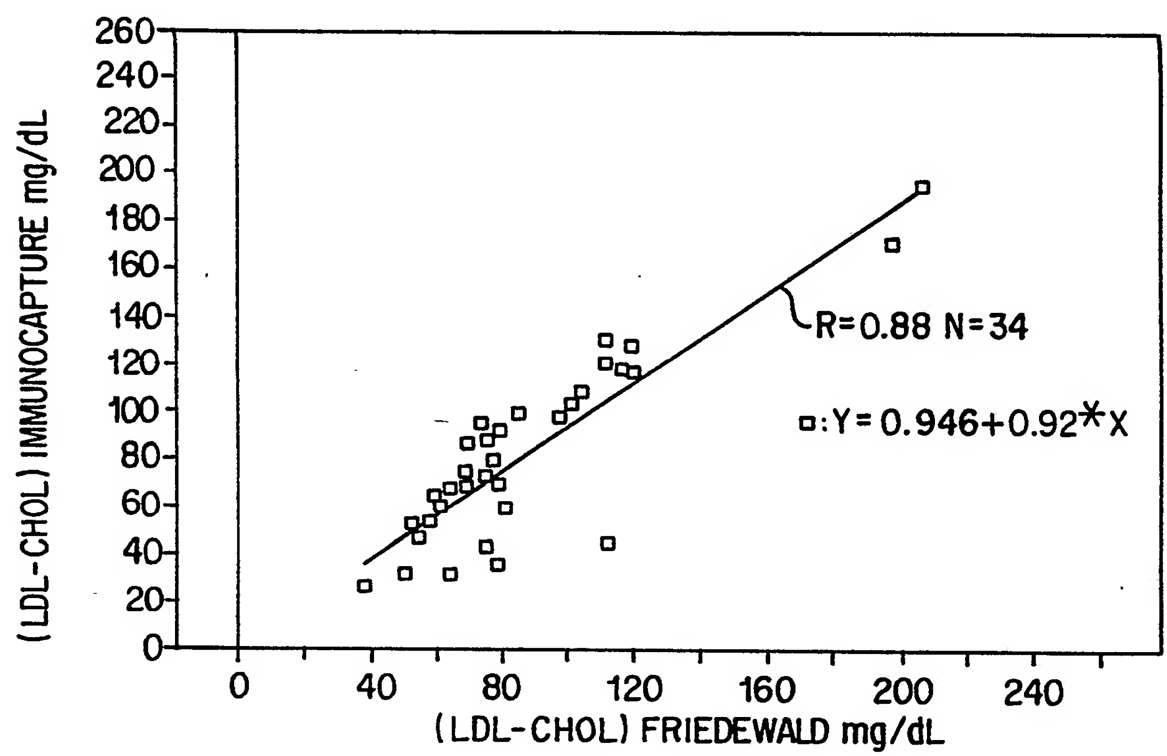



FIG.10B

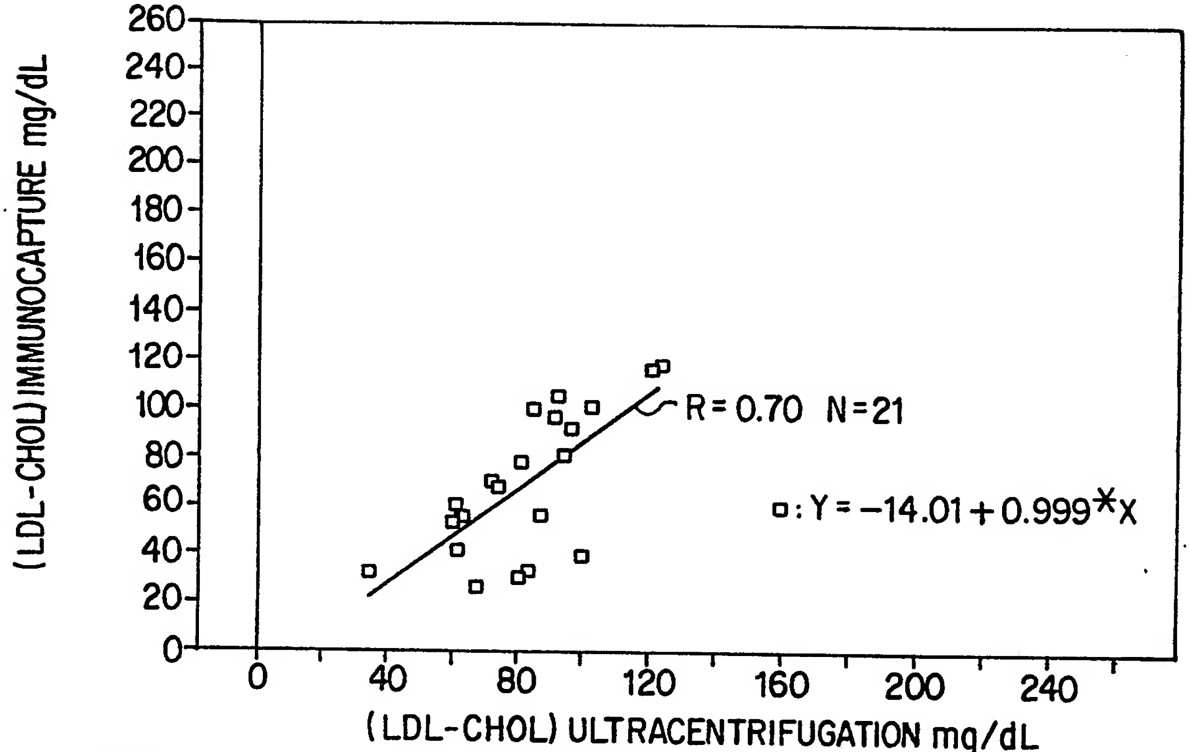



FIG. 11A

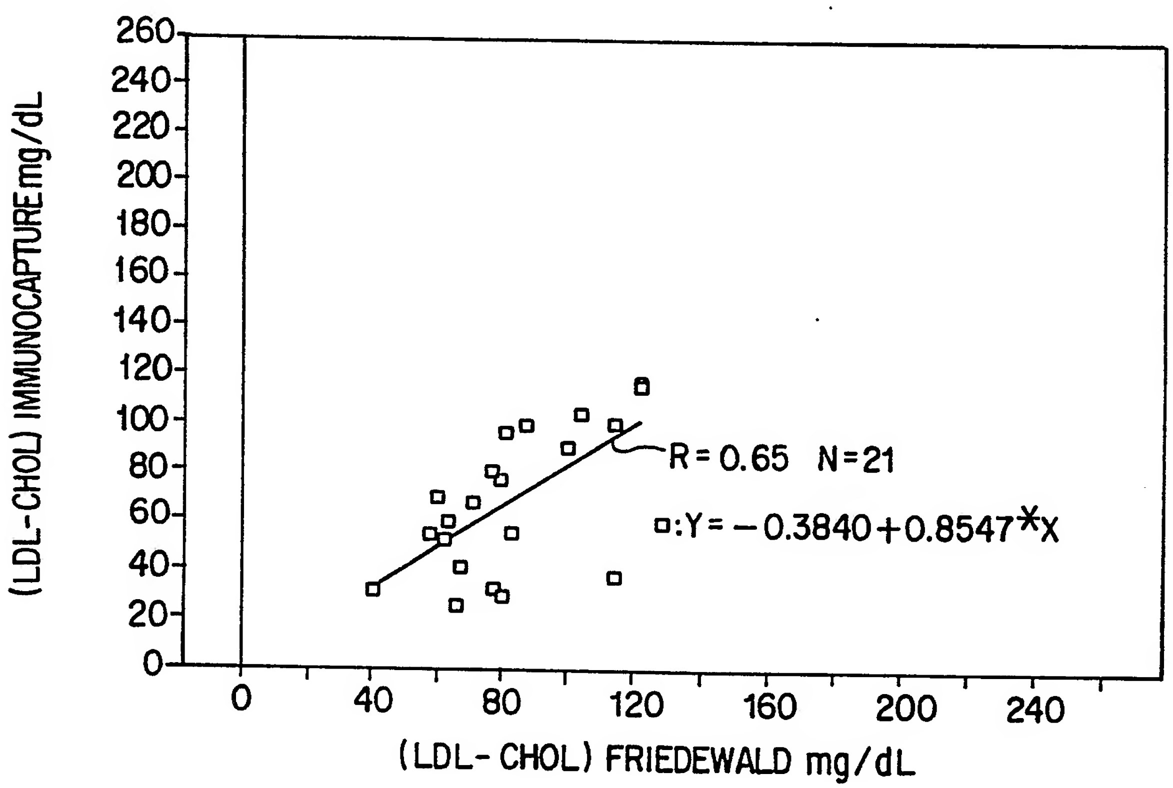



FIG. 11B

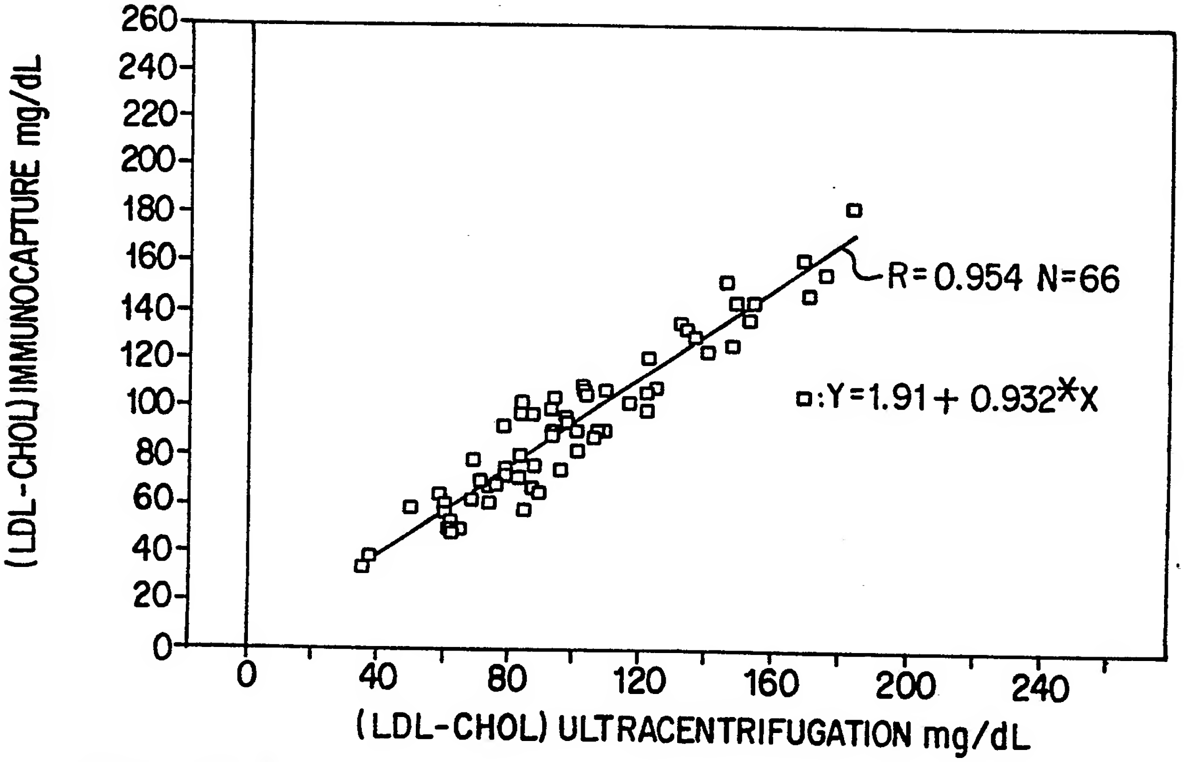



FIG. 12 A

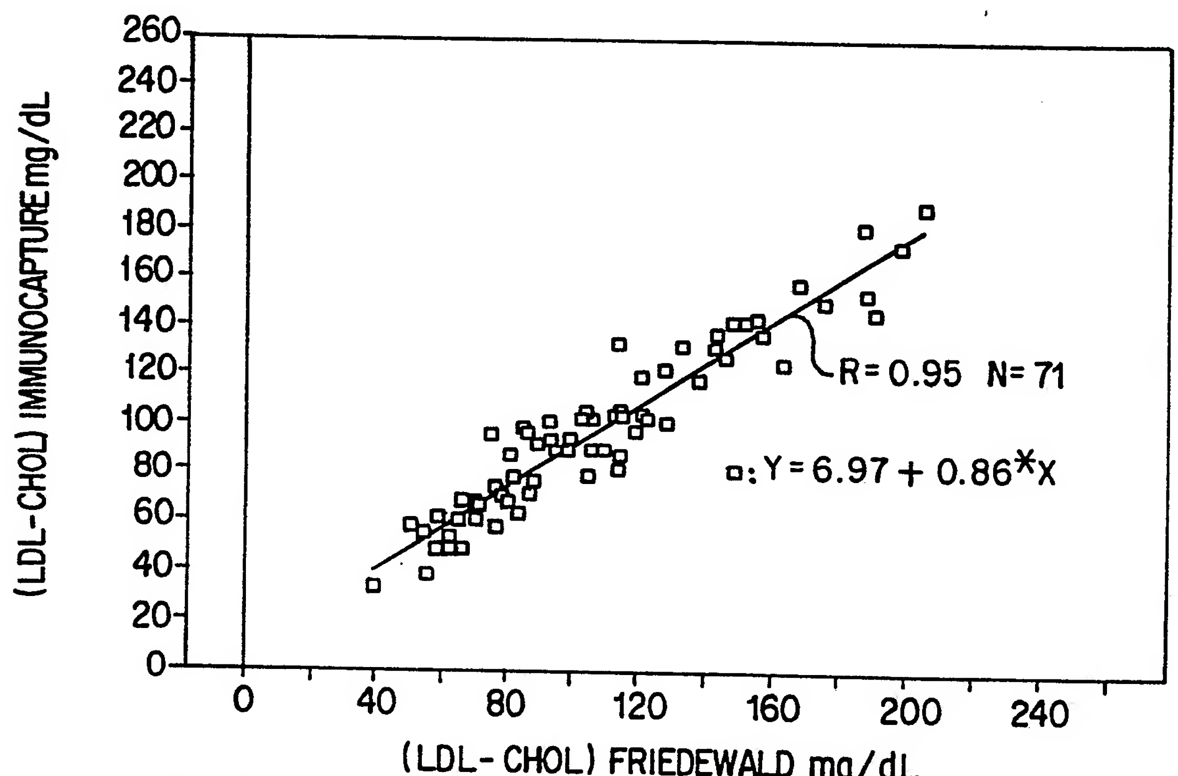



FIG. 12 B

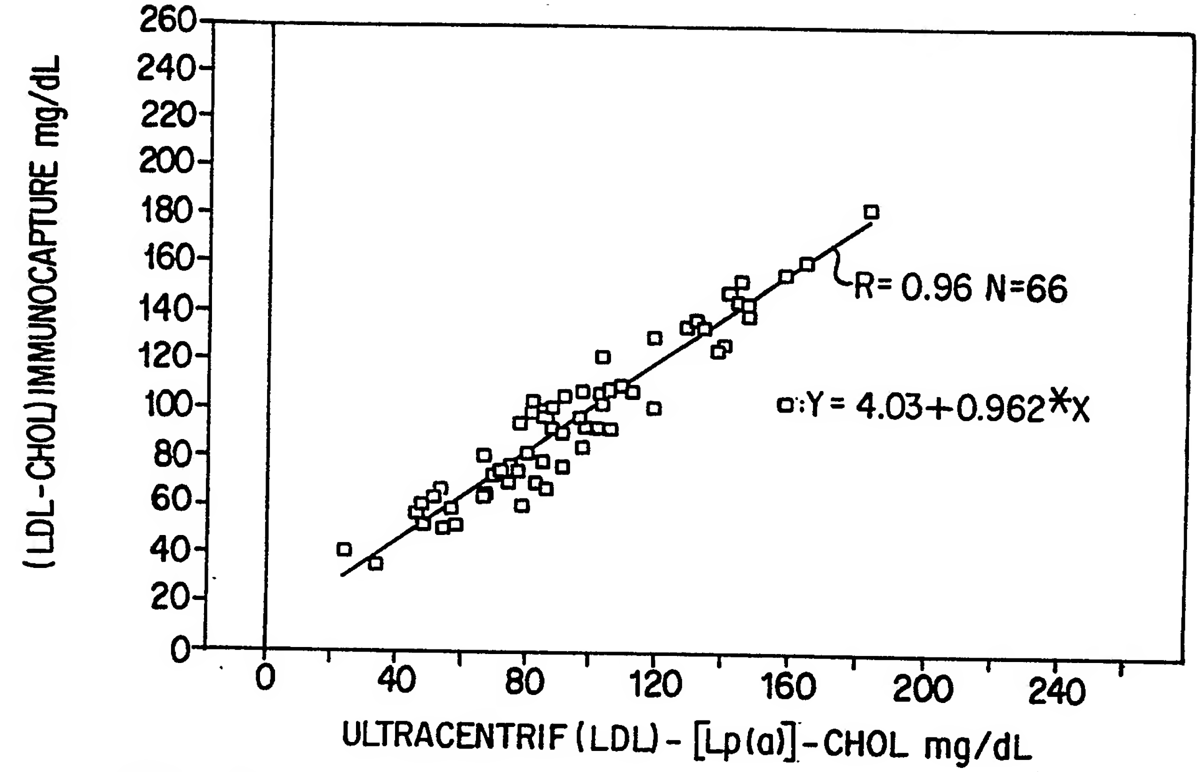



FIG. 13A

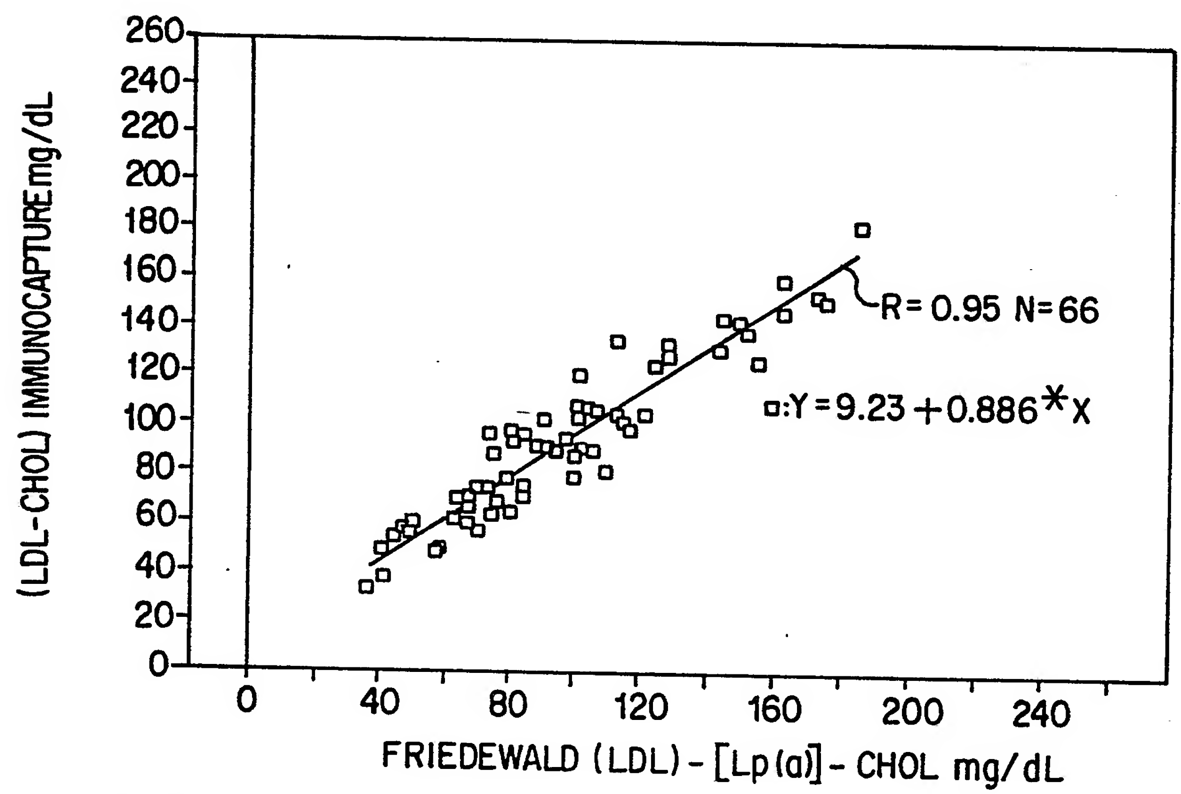



FIG. 13 B

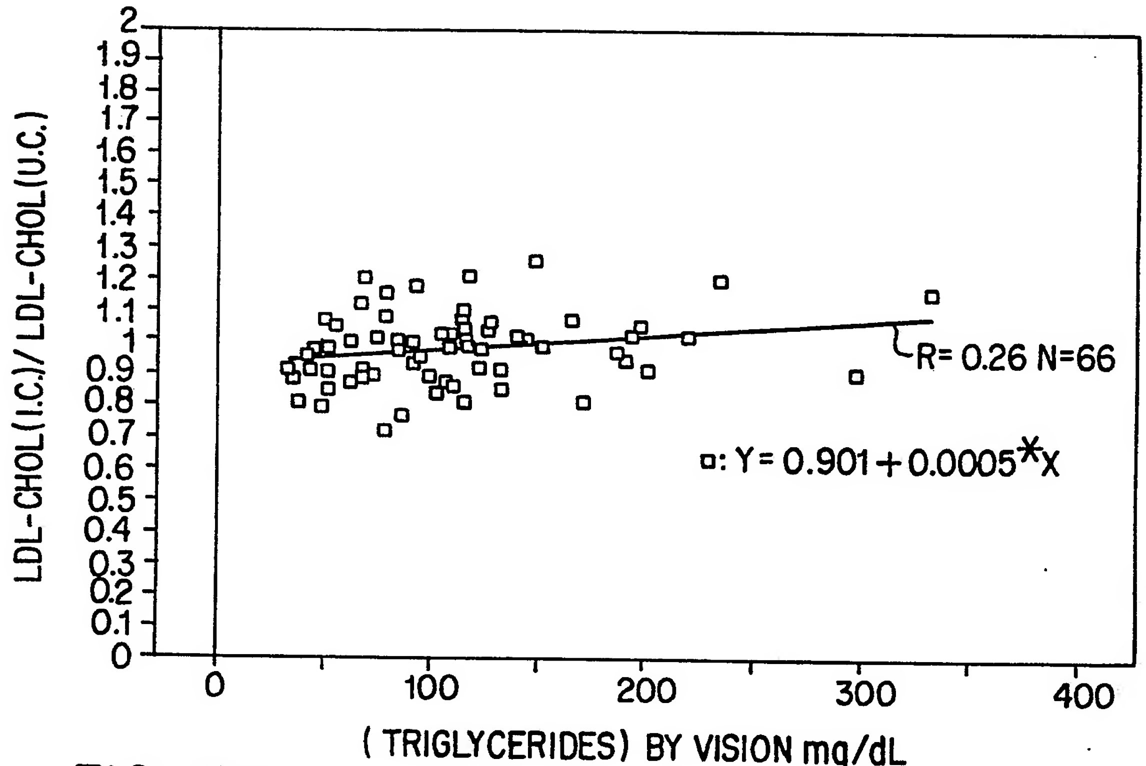



FIG. 14A

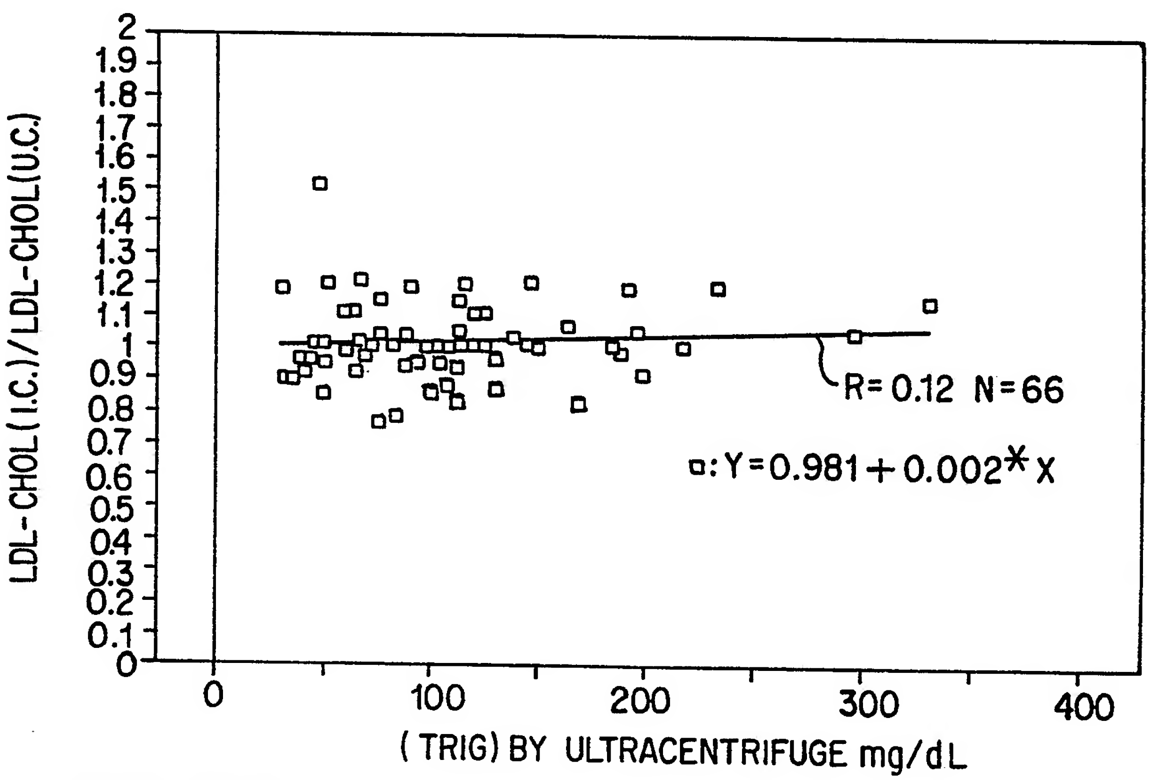



FIG 14 B

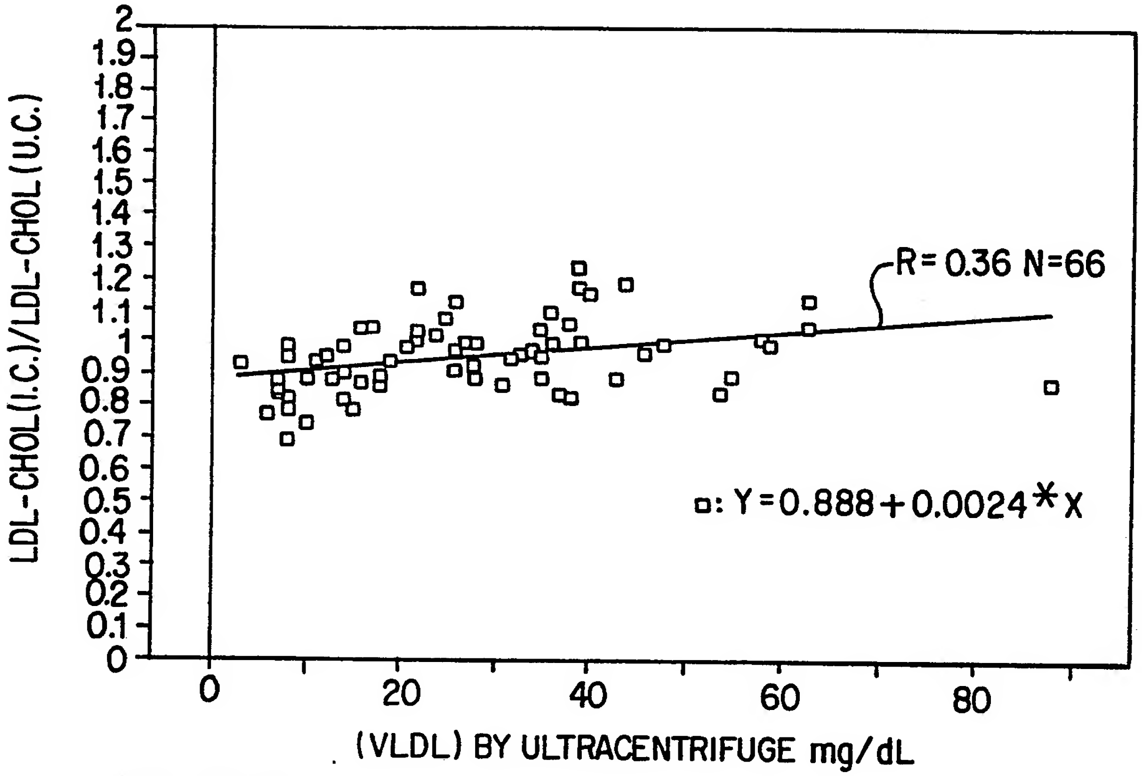



FIG.15A

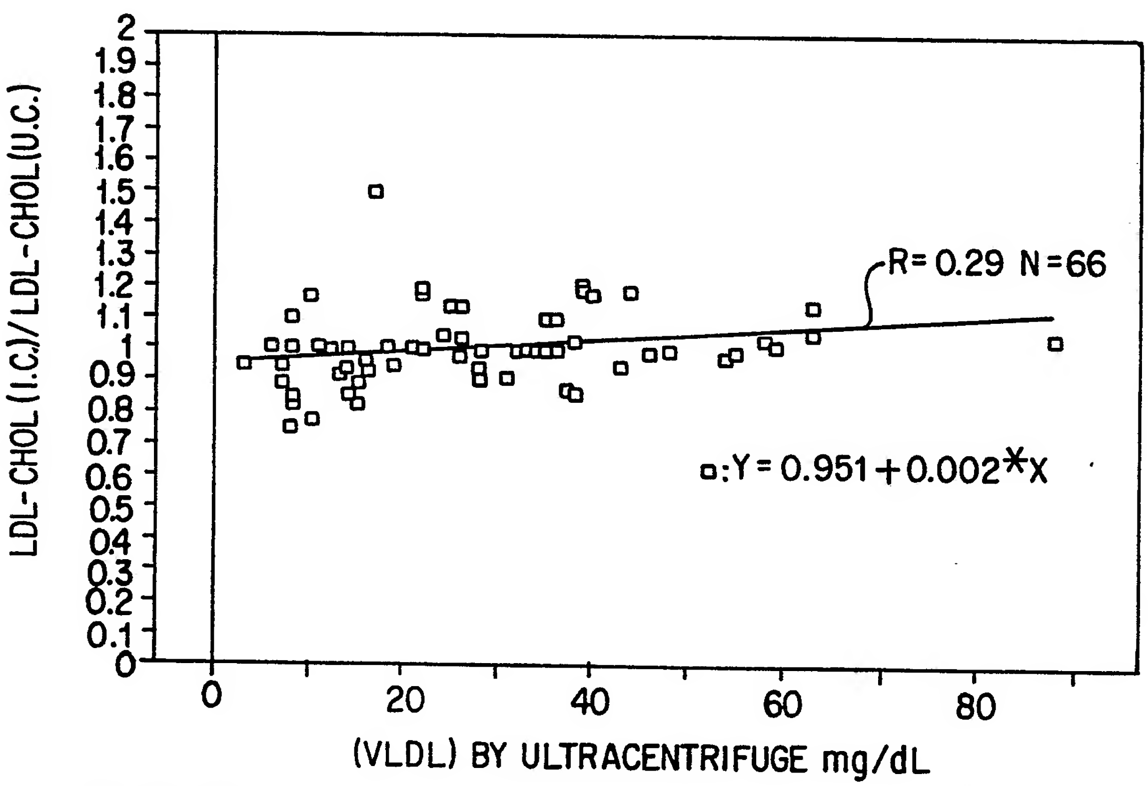



FIG.15B

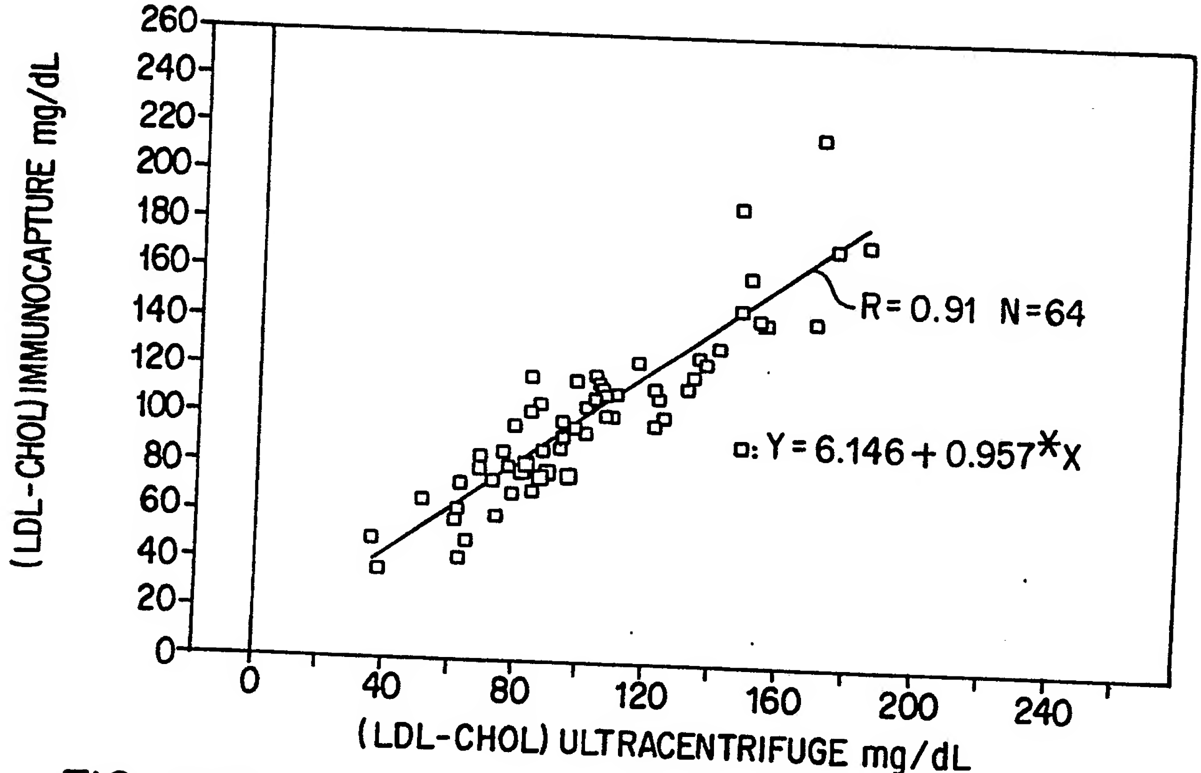



FIG. 16A

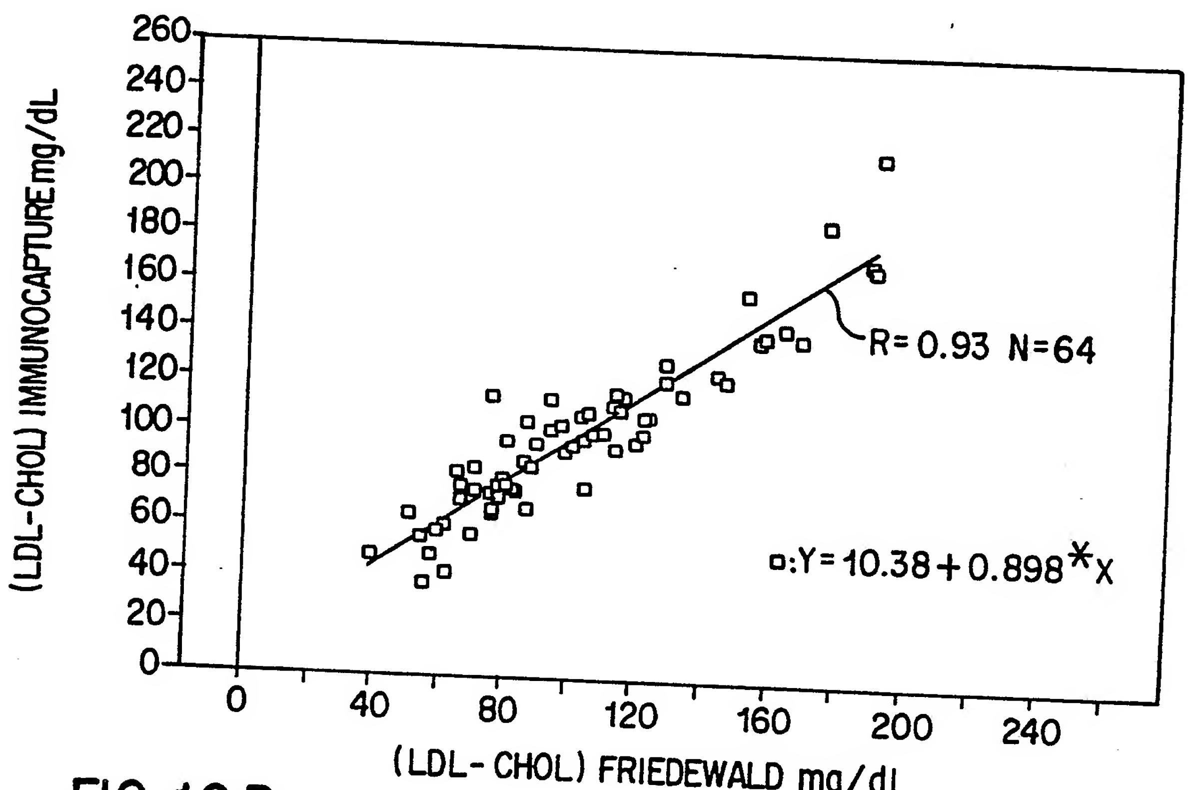



FIG. 16 B

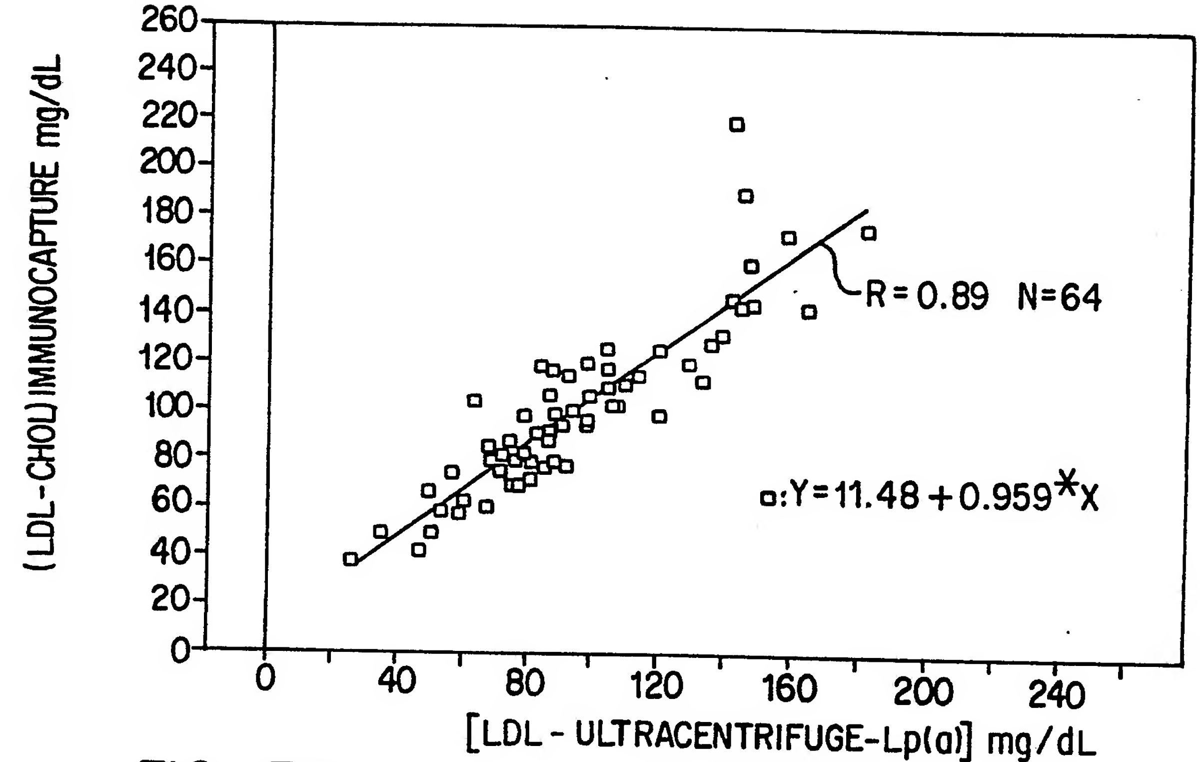



FIG. 17A

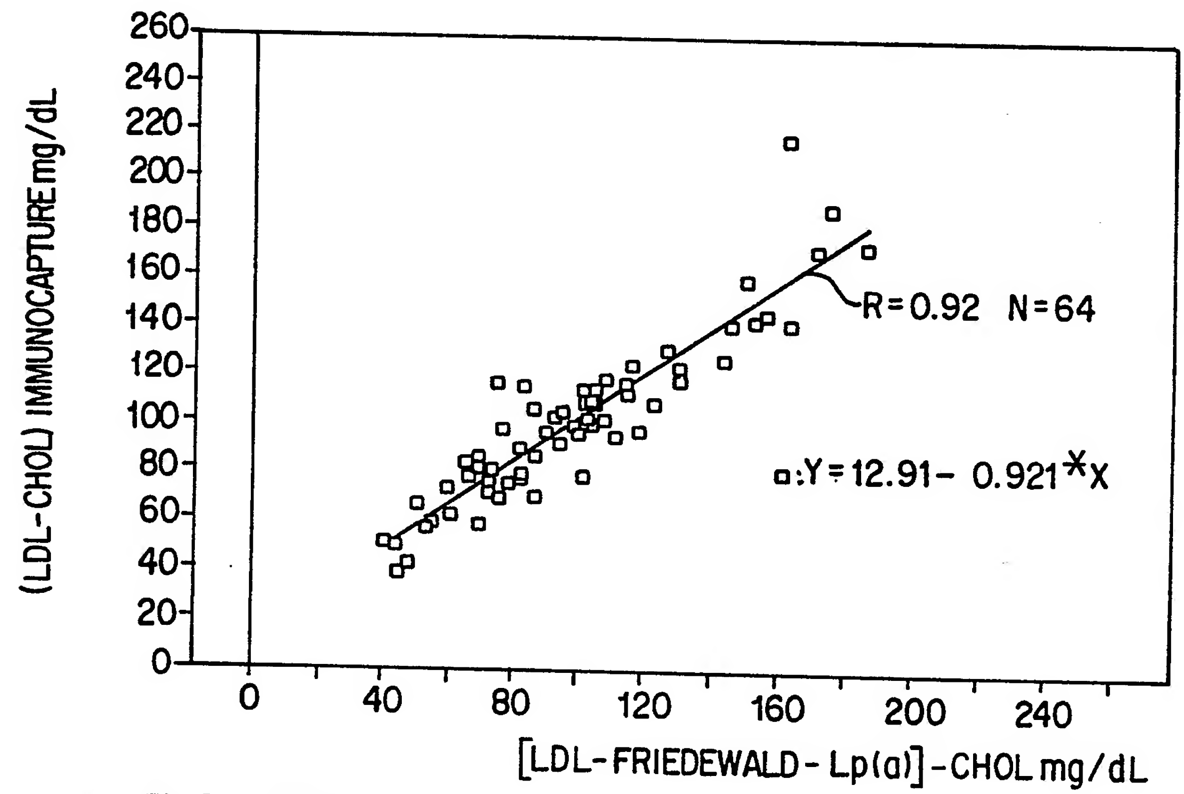



FIG. 17 B

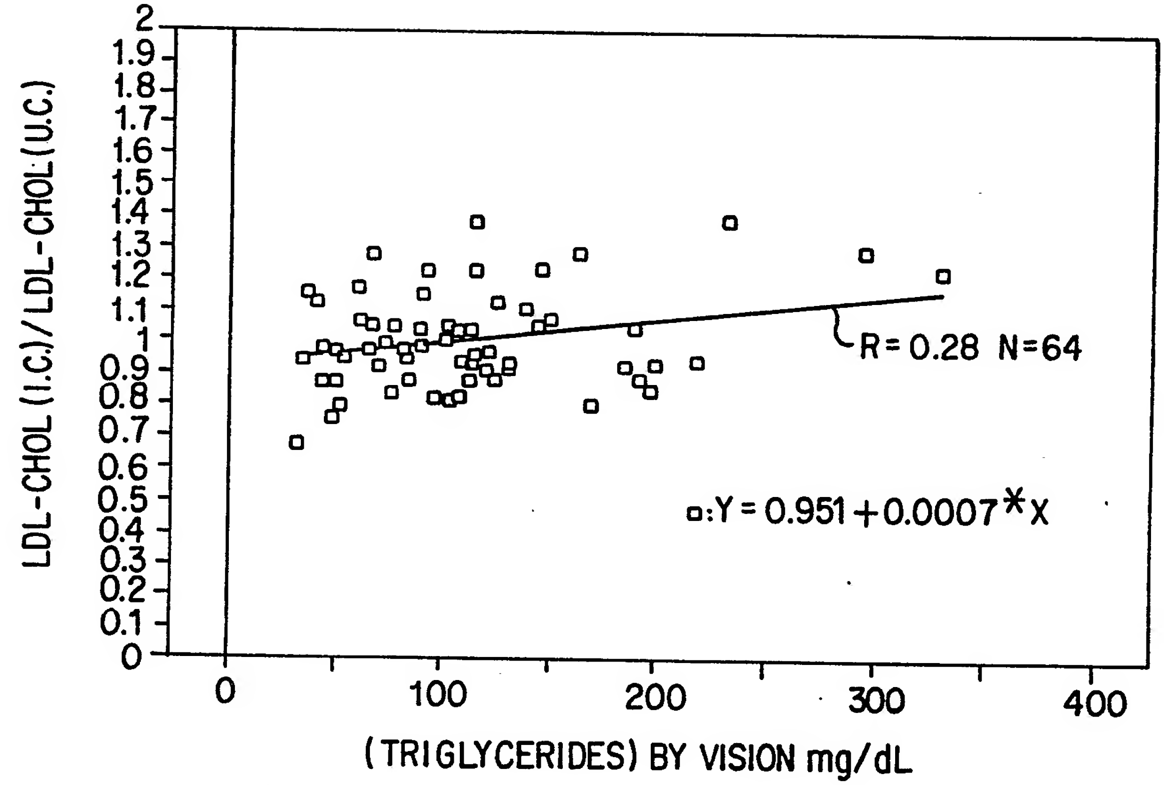



FIG.18A

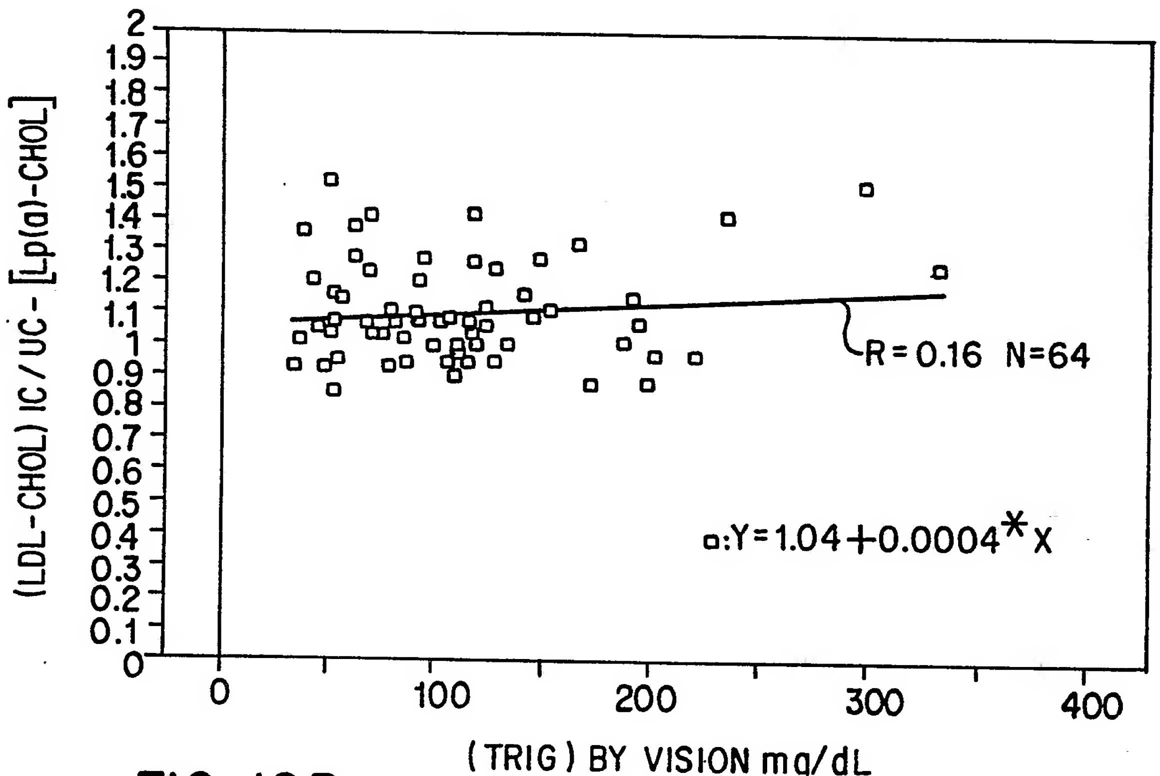



FIG. 18B

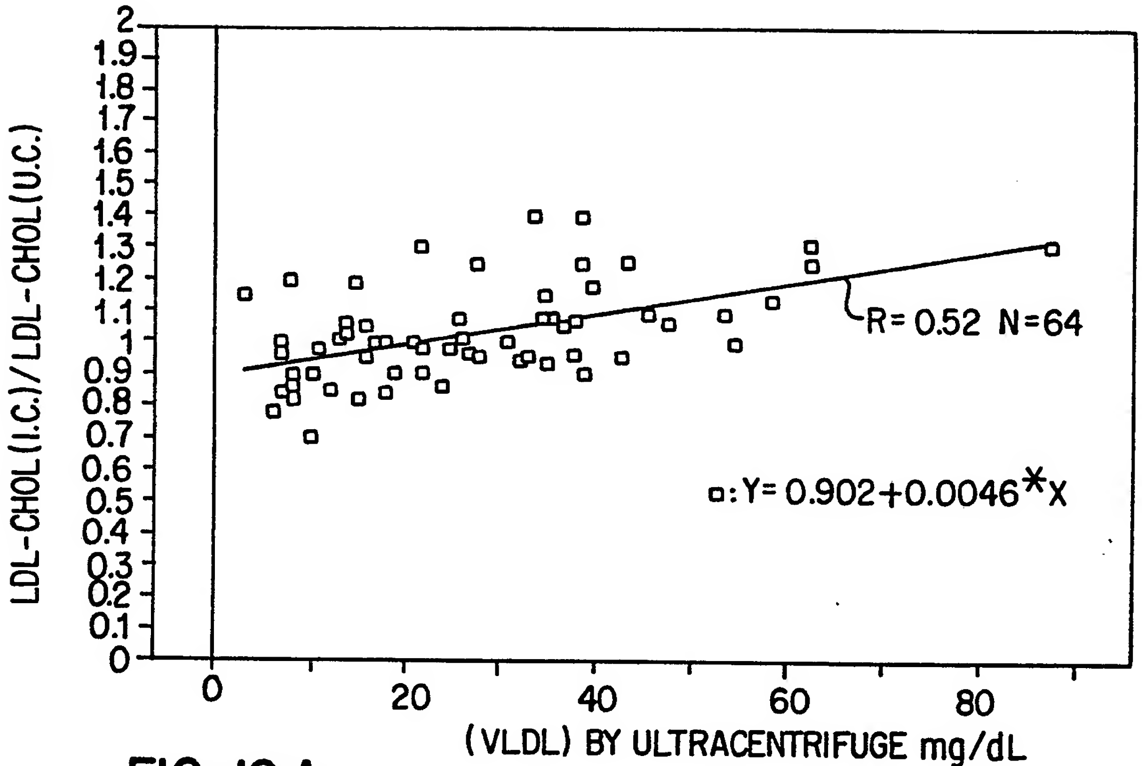



FIG. 19 A

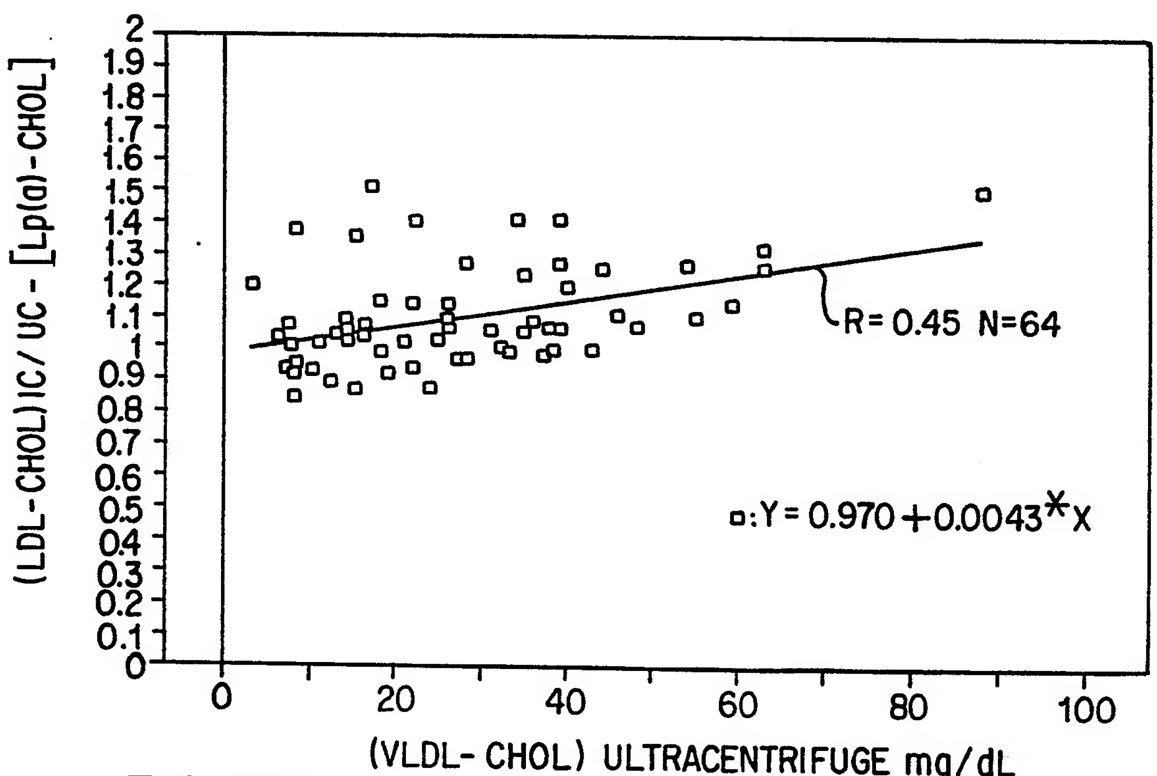



FIG. 19B

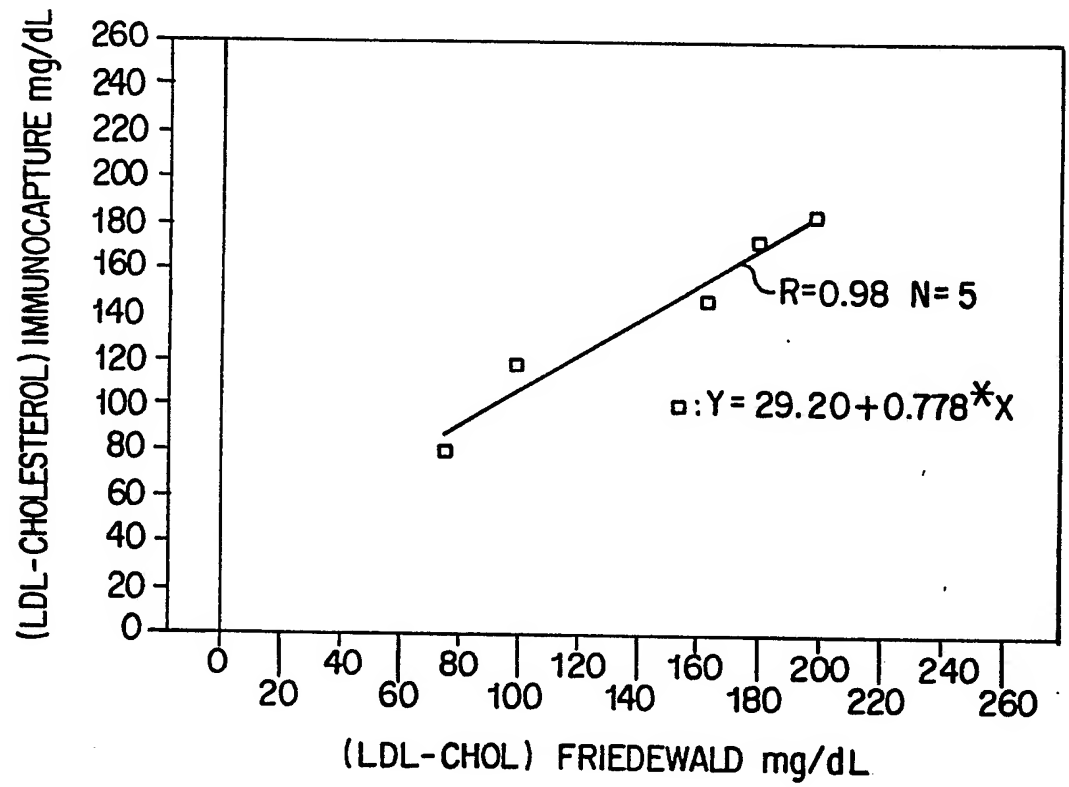

(LDL-CHOLESTEROL) IMMUNOCAPTURE mg/dL
260
240
220
200
180
160
140
120
100
80
60
40
20
0
0
20
40
60
80
100
120
140
160
180
200
220
240
260
(LDL-CHOL) FRIEDEWALD mg/dL
R=0.98 N=5
□:Y=29.20+0.778*X

FIG. 20

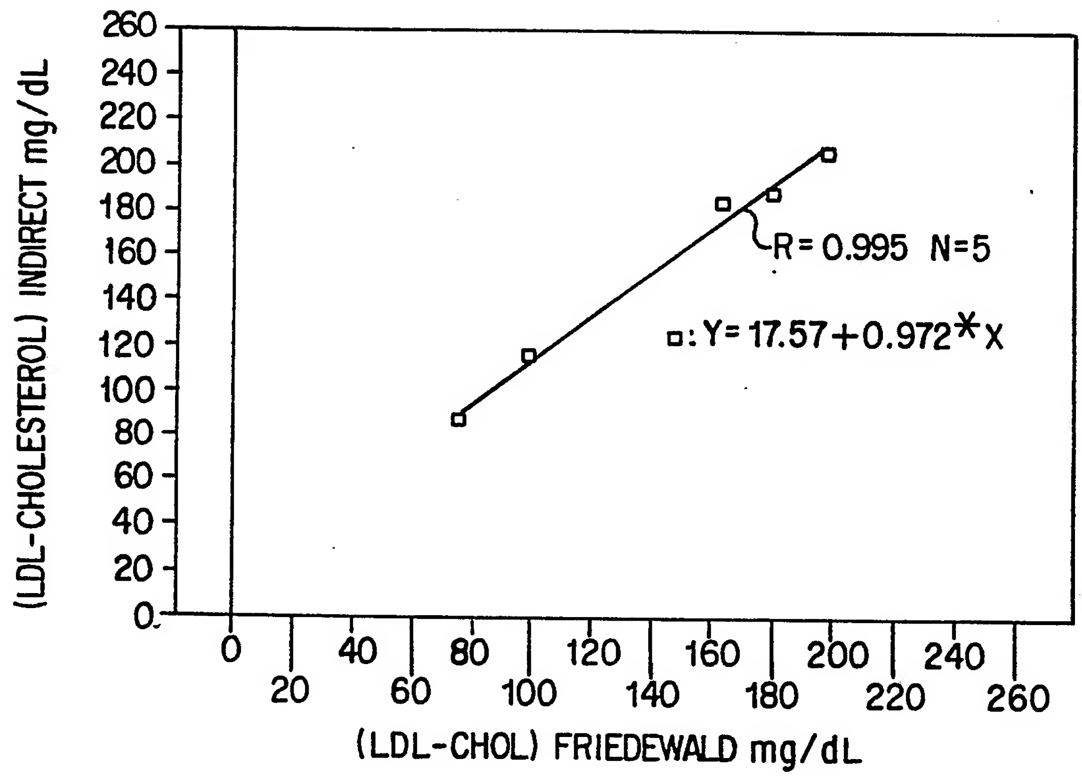
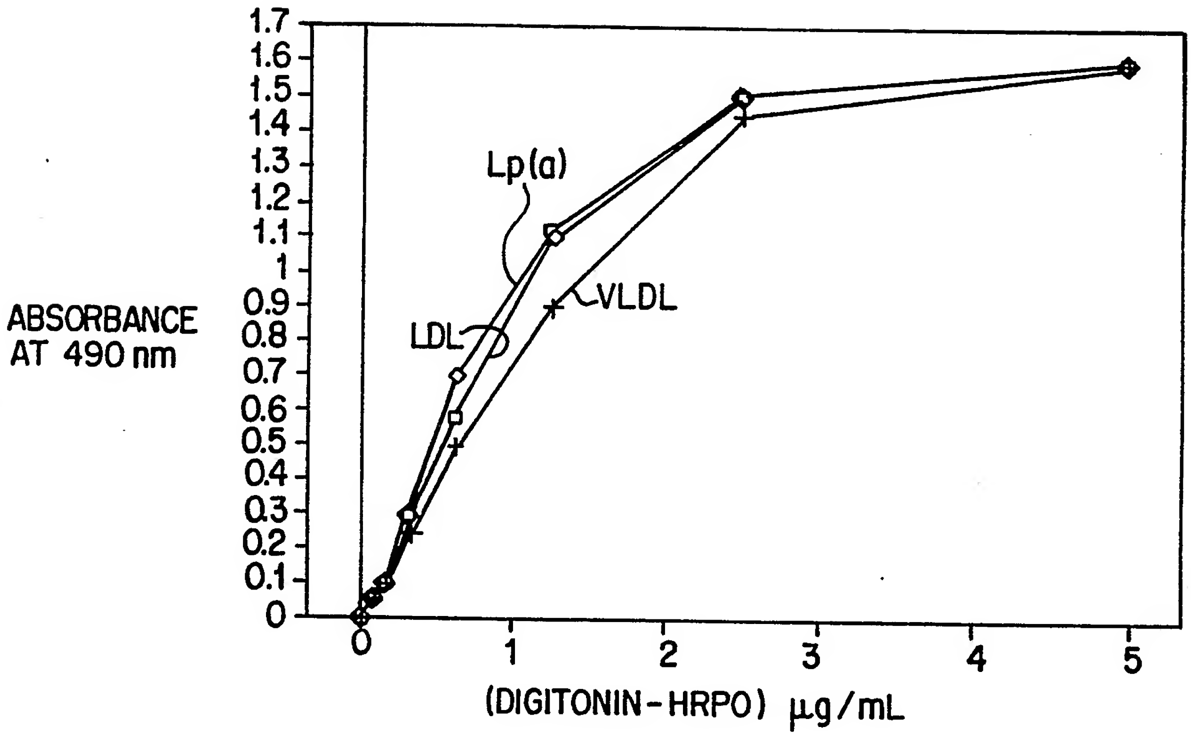

260
240
220
200
180
160
140
120
100
80
60
40
20
0
(LDL-CHOLESTEROL) INDIRECT mg/dL
R = 0.995 N=5
□: Y= 17.57+0.972*X
0
20
40
60
80
100
120
140
160
180
200
220
240
260
(LDL-CHOL) FRIEDEWALD mg/dL

FIG. 21



1.7
1.6
1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 490 nm
Lp(a)
LDL
VLDL
0
1
2
3
4
5
(DIGITONIN-HRPO) µg/mL


FIG. 22

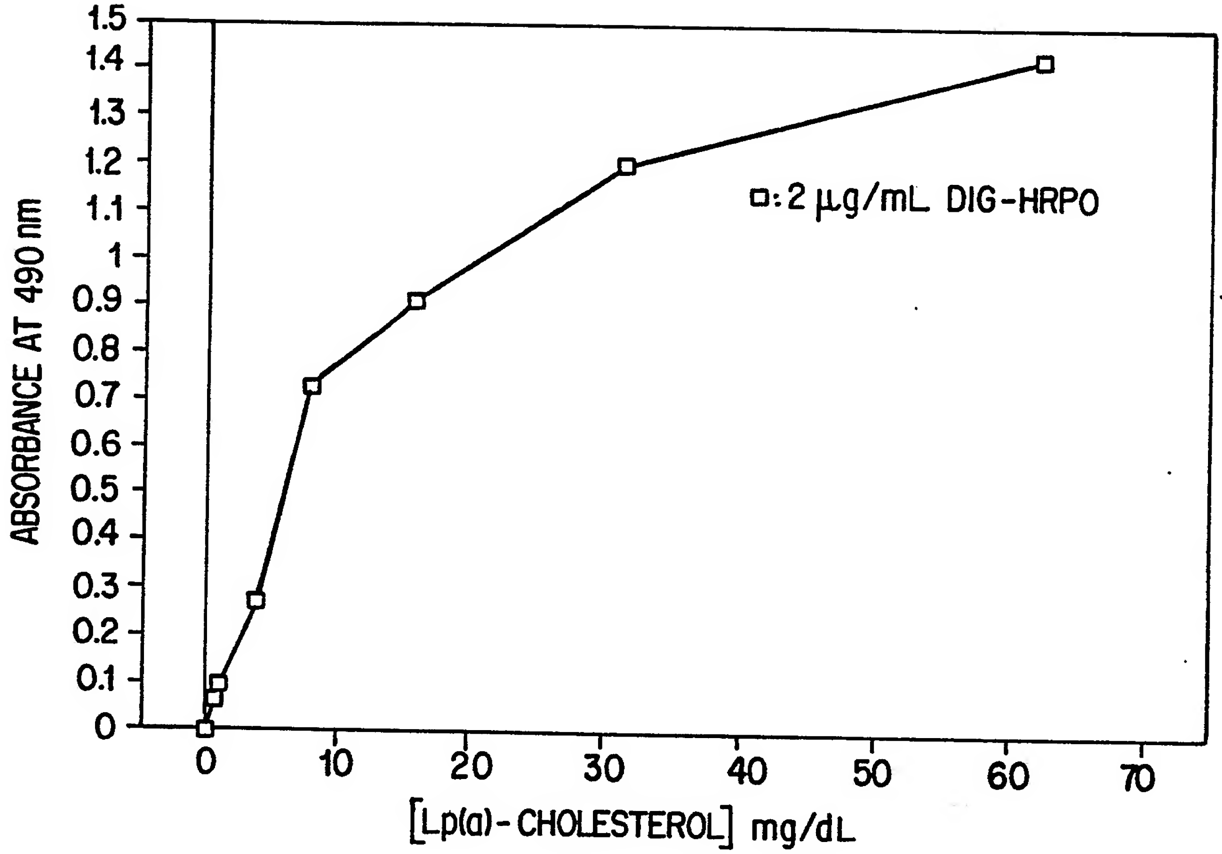

1.5
1.4
1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 490 nm
□: 2 μg/mL DIG-HRPO
0
10
20
30
40
50
60
70
[Lp(a)-CHOLESTEROL] mg/dL

FIG. 23

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/US93/02011

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) :C07K 15/28, 17/02, 17/14; G01N 33/92, 33/537, 33/544, 33/551
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 172.2, 240.27; 436/71, 524, 526, 528, 538, 548; 530/388.25, 389.3, 391.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE search terms: lipoprotein, cholesterol, antibody, assay, Lp(a), LDL; SEQUENCE search in A-GENESEQ-8, PIR 33, SWISS-PROT 23

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | US, A, 4,126,416 (SEARS) 21 November 1978, see col. 2, lines 14-21 and 51-56; col. 3, lines 1-11. | 1,8,14<br>2, 3, 5-7, 9,10,12,13, 15-19,26,27 |
| X<br>Y | US, A, 4,698,298 (DEDIEU et al) 06 October 1987, col. 1, lines 47-51; col. 2, line 58 through col. 3, line 32; col. 4, lines 57-68; col. 11, lines 30-68; col. 13, lines 21-54. | 20,21,23<br>22 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 17 May 1993 | 07 JUN 1993 |
| Name and mailing address of the ISA/US<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231 | Authorized officer<br>NANCY PARSONS |
| Facsimile No. NOT APPLICABLE | Telephone No. (703) 308-0196 |

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X<br>Y | CLINICA CHIMICA ACTA, Volume 191, issued 1990, M. LaBelle et al, "Increased immunoreactivity of apolipoprotein B epitopes during prolonged storage of low density lipoproteins", pages 153-160, especially pages 155,156. | 24,25<br>22 |
| X<br>Y | NATURE, Volume 323, issued 23 October 1986, T.J. Knott et al, "Complete protein sequence and identification of structural domains of human apolipoprotein B", pages 734-738, especially page 737. | 24,25<br>22 |
| Y | US, A, 4,619,895 (CUBICCIOTTI et al) 28 October 1986, col. 4; col. 10, lines 15-34; col. 14, lines 34-68; col. 15, lines 24-31. | 2,3,5-7,12, 13, 15-19, 26, 27 |
| Y | US, A, 4,935,147, (ULLMAN et al) 19 June 1990, col. 3, lines 56-64; col. 4, lines 23-35; col. 7, lines 25-28 and 42-44; col. 10, lines 34-46; col. 11, line 65 through col. 12, line 3; col. 13, lines 6-8; col. 17, lines 1-5. | 9,10 |
| Y | US, A, 4,885,256 (ALVING et al) 05 December 1989, columns 2 and 8. | 5,12,13 |

INTERNATIONAL SEARCH REPORT

| International application No. |
|---|
| PCT/US93/02011 |

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/7.1, 172.2, 240.27; 436/71, 524, 526, 528, 538, 548; 530/388.25, 389.3, 391.1